Integrated Master in Bioengineering

The Impact of Siloxanes on Wastewater Treatment Plants

Master's Thesis

of

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Resumo

Os metilsiloxanos voláteis (VMSs) são poluentes que, dado o seu caráter único, são amplamente utilizados em produtos de cuidado pessoal. Estes compostos são voláteis e são geralmente descarregados "down-drain", sendo recebidos em estações de tratamento de águas residuais (ETARs), bem como descarregados para o meio ambiente. Devido à sua natureza lipofílica, os VMSs têm tendência a absorver na lama e dado à suspeita da toxicidade destes compostos, é importante perceber a que níveis estão presentes na linha de lamas das ETARs. Quando a lama é anaerobiamente digerida, a maioria destes compostos migra para o biogás formado. No entanto, quando o biogás é queimado para produzir energia, podem ocorrer danos nos motores. Uma atenção especial deve também ser dada à lama estabilizada, que pode ser usada na agricultura como fertilizante, devido ao seu elevado conteúdo de nutrientes. Portanto, neste projeto, a presença de sete VMSs (L3, L4, L5, D3, D4, D5 eD6) foi estudada na linha de lamas da ETAR de Matosinhos, Portugal.

Para determinar as concentrações de VMSs nas lamas, foi desenvolvido um método analítico baseado na extração sólido-líquido acoplado à cromatografia gasosa com espectrometria de massa (GC-MS). A metodologia proposta foi validada, sendo alcançada uma gama de linearidade entre 5 e 1500 µg/L. Os limites de deteção (LODs) dos compostos estudados variaram entre 0,0004 (D5) e 0,47 ng/g ms (L3), enquanto os limites de quantificação (LOQs) variaram entre 0,001 (D5) a 1,60 ng/g ms (L3). A exatidão foi determinada através de testes de recuperação e variou entre 69±6% a 124±10% (considerando uma média dos diferentes níveis de fortificação). A recuperação média global foi de 93±15%. A precisão (repetibilidade e precisão intermédia) também foi avaliada e foram obtidos desvios-padrão relativos abaixo de 30%, o que é satisfatório neste tipo de matriz.

Foi também definido um esquema de amostragem na ETAR de Matosinhos, através da recolha de lamas do decantador primário, espessamento gravitacional, reator biológico, espessamento mecânico, tanque de lamas mistas, digestor anaeróbico e centrífugas de desidratação. Nas lamas primárias, as concentrações médias variaram entre 1008 (quarta-feira) e 2733 ng/g ms (segunda-feira). Após espessamento, as concentrações variaram entre 917 na quinta-feira a 2770 ng/g ms na segunda-feira. Nas lamas secundárias, concentrações superiores foram obtidas, variando no reator biológico de 4873 a 8059 ng/g ms. No processo de espessamento, as concentrações diminuíram ligeiramente (4549-6242 ng/g ms). A lama digerida registou uma diminuição nas concentrações (2999 a 6791 ng/g ms). Desta unidade ao processo de desidratação, as concentrações aumentaram de 24927 para 76412 ng/g ms. 98% dos siloxanos detetados em todas as amostras de lama foram cíclicos. O D5 foi o composto predominante em todas as amostras de lama, e a sua contribuição variou de aproximadamente 57% no espessador gravitacional para 82% no espessador mecânico.

Mais estudos sobre o destino dos VMSs devem ser desenvolvidos de modo a definir que quantidade é absorvida ou biodegradada nas lamas, volatilizada no ar ou mesmo a quantidade libertada no biogás.

Palavras-chave: metilsiloxanos voláteis, down-the-drain, persistência, ETAR, absorção, lama, GC-MS.

Abstract

Volatile methylsiloxanes (VMSs) are pollutants, that due to its unique character, are widely employed in personal care products. These compounds are volatile and they are usually discharged down-drain, being received in wastewater treatment plants (WWTPs), as well as released to the environment. Due to their lipophilic nature, VMSs usually adsorb on sludge and since these compounds are suspected of being toxic it is important to understand at which levels they are present in the sludge line of WWTPs. When sludge is anaerobic digested, most of these compounds normally migrate to the formed biogas. However, when biogas is burned to produce energy, damages may be provoked in the engines. Special attention should also be given to the stabilized sludge, which may be also use in agriculture as fertiliser due to the high content of important nutrients. Therefore, in this project, the presence of seven VMSs (L3, L4, L5, D3, D4, D5 and D6) was assessed in the sludge line from the WWTP of Matosinhos, Portugal.

To assess VMSs concentration in the sludge, an analytical method based on solid-liquid extraction coupled to gas chromatography-mass spectrometry (GC-MS) was developed. The proposed methodology was validated and a linearity range between 5 and 1500 µg/L was achieved. The limits of detection (LODs) of the studied compounds ranged between 0.0004 (D5) and 0.47 ng/g dw (L3), while limits of quantification (LOQs) ranged between 0.001 (D5) to 1.60 ng/g dw (L3). Accuracy was assessed by recovery tests and ranged from 69±6% to 124±10% (considering a mean of the different levels of spiked concentrations). A global mean recovery of 77±2% was achieved. Precision (intra- and inter-day) was also assessed, and relative standard deviations below 30% were found, which is satisfactory for this type of matrix.

A sampling scheme was defined in the WWTP of Matosinhos, collecting sludge from the primary settler, gravitational thickening, biological reactor, mechanical thickening, mixed sludge tank, anaerobic digester and dehydration centrifuges. In the primary sludge, mean concentrations ranged between 1008 (Wednesday) to 2733 ng/g dw (Monday). In the thickened samples, concentrations ranged from 917 on Thursday to 2769.9 ng/g dw on Monday. In the secondary sludge, higher concentrations were achieved, varying in the biological reactor from 4873 to 8059 ng/g dw. In the thickening process, concentrations slightly decreased (4549 - 6242 ng/g dw). The digested sludge also registered a decrease on concentrations (2999 to 6791 ng/g dw). From this unit to the dewatering process, concentrations increased ranging from 2493 to 7642 ng/g dw. 98% of the siloxanes detected in all the sludge samples were cyclic. The predominant compound in all the sludge samples was D5, where its contribution varied from approximately 57% in the gravitational thickener to 82% in the mechanical thickener.

Further studies on the fate of VMSs should be carried out in order to understand the quantity absorbed or biodegraded in the sludge, volatilized or even the amount released with the biogas.

Keywords: volatile methylsiloxanes, down-the-drain discharges, WWTP, sorption, sludge, GC-MS.

Declaration

I hereby declare, on my word of honour, that this work is original and that all non-original contributions were properly referenced with source identification.

Date: Monday, June 25, 2018

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(Joana Correia da Silva)

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Notation and Glossary

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a	Slope	
Acet	Acetone	
ACN	Acetonitrile	
AS	Aerobic sludge	
BDL	Below detection level	
BOD_5	5-day Biochemical Oxygen Demand	
C	Concentration	ng/g dw
cVMS	Cyclic volatile methylsiloxane	
D3	Hexamethylcyclotrisiloxane	
D4	Octamethylcyclotetrasiloxane	
D5	Decamethylcyclopentasiloxane	
D6	Dodecamethylcyclohexasiloxane	
DCM	Dichloromethane	
dw	Dry weight	
DS	Digested sludge	
EC	European Community	
EPA	Environmental Protection Agency	
EPS	Extracellular polymeric substances	
EtAc	Ethyl acetate	
ES	Excess sludge	
FAO	Food and Agriculture Organization	
FID	Flame Ionization Detector	
GC	Gas chromatography	
GC-FID	Gas chromatography- flame Ionization detector	
GC-MS	Gas chromatography-mass spectrometry	
HCH	Heavy hydrocarbons and halogenated compounds	
Hex	n-hexane	
IC_{50}	Half-maximum inhibitory concentration	
IIM	Insoluble inorganic matter	
IOM	Insoluble organic matter	

IS	Internal standard	
IUPAC	International Union of Pure and Applied Chemistry	
L3	Octamethyltetrasiloxane	
L4	Decamethyltetrasiloxane	
L5	Dodecamethylpentasiloxane	
LLE	Liquid-liquid extraction	
LOAEC	Lowest-observed-adverse-effect-concentration	
LOD	Limit of detection	
Log K_{OW}	Logarithm of the octanol/water partition coefficient	
LOQ	Limit of quantification	ng/g dw
LRAT	Long range atmospheric transport	
lVMS	Linear volatile methylsiloxane	
m/z	Mass-to-charge ratio	
M4Q	Tetrakis(trimethylsilyloxy)silane	
MS	Mass spectrometer	
n	Number of repetitions/replications	
NA	Not available	
ND	Not detected	
NOEC	Non-observed-effect-concentration	
p	Risk value that the investigator is willing to take	
PBT	Persistent, bioaccumulative and toxic	
PCP	Personal care product	
PDMS	Polydimethylsiloxane	
PS	Primary sludge	
r	Pearson correlation coefficient	
R^2	Coefficient of determination	
RAS	Return activated sludge	
Rec	Recovery rate	%
RF	Response factor (Peak area of the compound/Peak area of the internal standard)	
RSD	Relative standard deviation	%
RT	Retention time	minutes
S/N	Signal-to-noise ratio	

s_a	Standard deviation of the slope
SIS	Selected ion storage
SIM	Soluble inorganic matter
SLE	Solid-liquid extraction
SPE	Solid-phase extraction
SOM	Soluble organic matter
SRT	Sludge Retention time
TOC	Total organic carbon
TS	Thickened sludge
TWAS	Thickened waste activated sludge
US EPA	United States Environmental Protection Agency
USE	Ultrasound extraction
VMS	Volatile methylsiloxane
vPvB	Very persistent, very bioaccumulative
VOC	Volatile organic compound
ww	Wet weight
WWT	Wastewater treatment
WWTP	Wastewater treatment plant

1 Project Presentation

EFACEC is a Portuguese company founded almost 70 years ago and present in more than 65 countries, with a strong exporter profile. Its origin took place in the “A Moderna” Sociedade de Serração Mecânica, in 1905, only later in 1948 becoming EFACEC, the largest National Electricity Group of Portuguese capitals. It has more than 2330 employees and several dozen open recruitment processes. In 2015, the company invoiced approximately €430 million, exporting about 76% of its production (EFACEC, 2017). EFACEC’s portfolio of activities offers solutions in the areas of Energy, Engineering and Services and Transport and Logistics, sustaining an increasingly systemic/integrative approach, satisfying the current market needs and monetizing several Group's values. In the Environment area, EFACEC Environment S.A., covers mainly two major areas: water (in the design and implementation of water and effluent treatment systems) and air (in the design of dedusting, flushing, heating, ventilation and air conditioning systems). Through a highly qualified technical staff, with the necessary know-how, the EFACEC Group offers integrated solutions ranging from conception and design to the realization and operation of systems. In this way, it contributes strongly to the evolution of the environmental policy, and consequently, to the placement of the Country, within an internationally important position, regarding the quality of life and well-state of its populations (Aicep Portugal Global, 2013; EFACEC, 2017).

The focus of this project will be the environmental area, oriented towards the effluent treatment from the Wastewater Treatment Plant (WWTP) of Matosinhos. In 1997, it was one of the pioneer municipalities of Portugal to construct a WWTP. The main goals of this WWTP is to receive and treat wastewater from the municipality of Matosinhos and 8 parishes from Vila do Conde, de-polluting the rivers and enhance the quality of the coastline, before discharging the wastewater directly to the environment, resorting to a secondary treatment. This investment was over €16 million, resulting in an improvement of the quality of water on the beaches of the Municipality. This WWTP was designed to respond to a population equivalent of 329 138 (Matos, 1996; Sisaqua, 2016). In order to assess whether or not the resulting treatment has a positive impact on the reduction of pollutants, this study will focus on the analysis and detection of a specific group, volatile methylsiloxanes (VMSs). As we shall see later, WWTPs have been aware of the problem associated with the presence of VMSs. In fact, the concern to reduce them has been notorious across the globe, due to either their bioaccumulative and toxic potential in the ecosystem, as well as by their presence accelerating the degradation process of the cogeneration biogas engines, causing extra expenses to the WWTPs. Therefore, EFACEC, facing the future and prepared for new and important challenges, thanks to its resilience and adaptability, but above all ceaseless capacity to innovate, wants to understand at which levels these pollutants are present in their facilities, to contribute to a more sustainable world (Matos, 1996; Sisaqua, 2016).

2 Introduction

2.1 Wastewater Treatment Plants (WWTPs)

A famous saying of Thales of Miletus, one of the seven wise men in Ancient Greece, in the explanation that postulated the creation of the World is “Water is the primary principle”. It is not only an essential resource for life, but is also used every day for production, leisure, to guarantee health, to preserve the environment and for the economic and social development. The environmental sector, in particular regarding public water supply services to the population, plays an important role in society, being the access to these services (and their quality) important aspects to the development of both countries and communities (Albuquerque et al., 2010).

Integrated systems of municipal wastewater treatment arose from the need of preserving the hydric resources and the environment, due to the increase of the pollution produced by population and industries (Santos, 2012). Nowadays, the potable water is mostly used for daily household activities, services and industries instead of being used as a beverage (European Comission, 2016). Therefore, this water may transport pollutants from those activities to the receiving water bodies, namely lakes, rivers or sea. That is why it is important to treat the wastewater, to obtain an effluent with minimum or no effect on the quality of the environment (Santos, 2012). Although WWTPs reduce the pollution load, they were not initially designed for the removal of some substances that have arisen recently as prone to have a negative impact on the environment, such as oil, pharmaceuticals, personal care products, etc. These pollutants can have an important repercussion in drainage systems, in WWTPs (they can inhibit biological treatments, create sludge with toxic and dangerous characteristics, etc.), as well as in the receiving means (BCRSD, 2016; Moura, 2009). So, the main purpose of WWTPs is, through a sequence of operations, to achieve and produce a harmless effluent that does not damage the receiving ecosystem and, if possible, to benefit the watercourses and the surrounding environment where it will be introduced. In order to avoid health issues from the use of water, since human population is part of the ecosystem, WWTPs intend to reduce and enable the elimination of pathogenic microorganisms, as well as other harmful substances (Santos, 2012; Villalón, 2015).

Commonly, wastewater can go through several treatment processes, as seen in the scheme of Figure 1, explained henceforth.

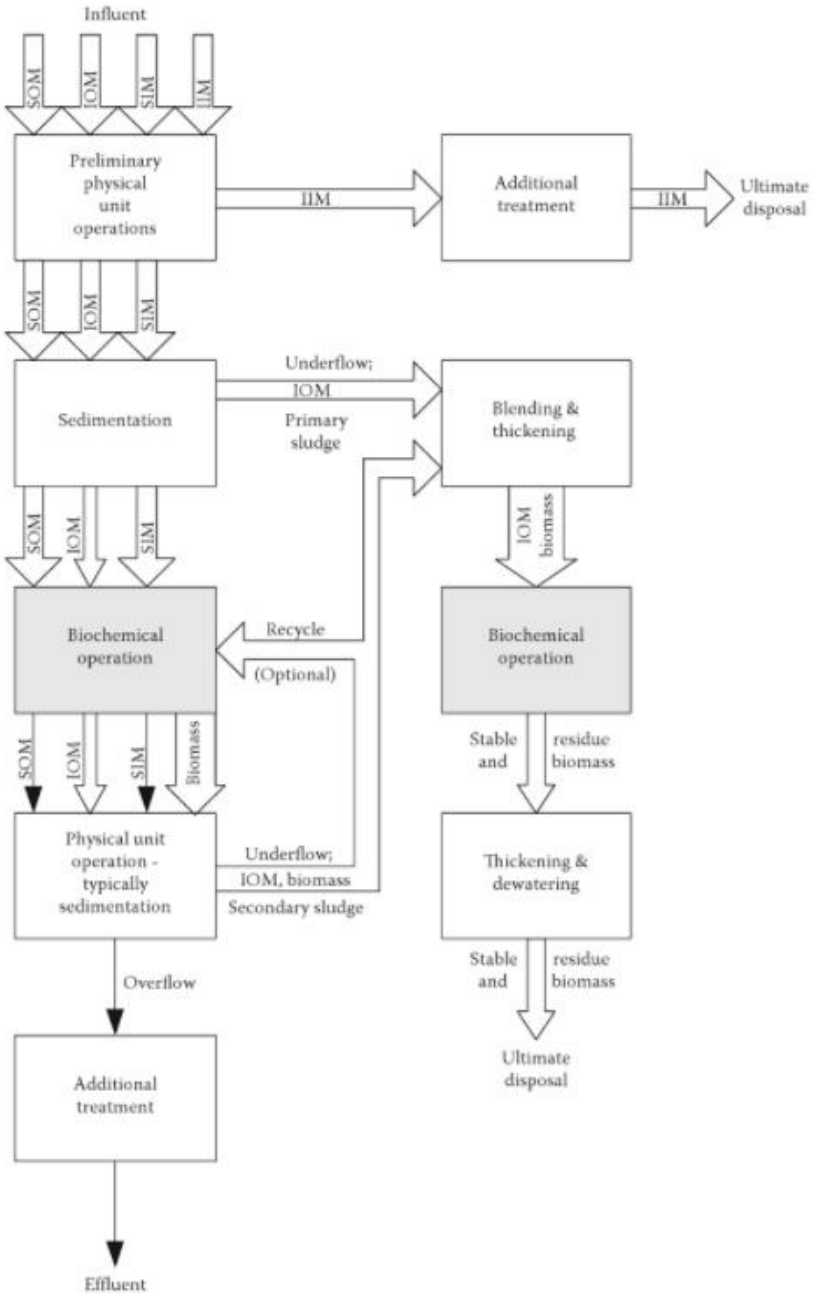


Figure 1 Typical wastewater treatment plant scheme (Grady et al., 2011).

2.1.1 Preliminary Treatment

The wastewater derived from domestic, commercial and industrial waste streams along with storm water run-off carry faecal matter, suspended and floating debris, containing grit and other inert solids. The preliminary treatment ensures a satisfactory quality of the final effluent and final sludge product and protects the other processes from malfunction associated with accumulation of debris, inorganic grit, excessive scum formation or loss of efficiency associated with grease or oil films or even fat accumulations, using a tank called grit chamber (Botelho, 2015; Butler et al., 1995).

2.1.2 Primary Treatment

The term “primary” is related with a primary sedimentation. Whereas the preliminary treatment separates inert or stable material, the primary sedimentation separates sludge with a high content of biodegradable organics, by means of a specific (anaerobic or aerobic) stabilization stage (Levy et al., 2011). Thus, primary treatment consists in separating the pollutant load from the wastewater with the use of primary settlers. This process, in some cases, can be helped with the addition of chemical agents that, through coagulation or flocculation, enable the production of flakes of bigger dimensions, facilitating their decantation. The reason why the preliminary and primary treatment are separated processes it is because it results in separated flows of inert and decomposable sludge, which is relevant when considering an anaerobic stabilization of primary sludge. Therefore, it is possible to produce energy, an additional resource, rather than having only a disposal extra treatment (Botelho, 2015; Levy et al., 2011; Moço, 2012).

2.1.3 Secondary Treatment

The wastewater is then piped from the sedimentation tank to the aeration tank. Air, or pure oxygen, is introduced into the tank, promoting the mix between the wastewater and the sludge. This allows an aerobic biological treatment, removing the dissolved organic matter of the effluent (Michigan Technological University, 2003). The biological treatment uses aerobic microorganisms (usually bacteria) that degrade the organic matter, converting it to carbon dioxide, water and energy. The final step of this treatment involves an additional settling process to remove more suspended solids in a secondary sedimentation tank, separating the sludge from the wastewater (Mongillo et al., 2000). Once the sludge has settled out, the water can have two courses: if the treatment is considered complete, it can be discharge into a lake, stream or in the ocean. If not, it passes through a tertiary, or advanced treatment. The same happens within the sludge. It either undergoes to a tertiary treatment or, if collected at the bottom, it can be then removed, dried and disposed of (Mongillo et al., 2000).

2.1.4 Tertiary Treatment

The tertiary treatment or advanced treatment is intended to remove all the pathogenic elements or, in some cases, some suspended or dissolved solids, organic matter, toxic substances and nutrients. The removal of nutrients, such as nitrogen and phosphorus (through denitrification and chemical precipitation, respectively), is relevant since they are responsible to potentiate the eutrophication of the receiving waters. Moreover, this step reduces 5-day biochemical oxygen demand (BOD5), providing the highest quality of wastewater effluent (EPA, 2002; Kadlec et al., 1995; OTA, 1981; Wang et al., 2007).

2.1.5 Sludge Treatment

During the wastewater treatment process, sludge is generated from the different operations and, according with the final destination, the treatment that it undergoes varies (Moço, 2012). The most used techniques to produce energy from sludge and to treat it are (Gurjar et al., 2017):

- **Composting:** intends to stabilize biologically the sludge (both non-digested and digested sludge), through an aerobic digestion of the organic matter, controlling also the pollution risks, to get a final product that can have an added value to agriculture, based on its composition (nutrient or organic value). This process, also decreases the water content, by generally using a temperature of around 60 °C, enriching the content of solids from 40 to 60% (Bresters et al., 1999);
- **Thermal drying:** the bases of the process is to evaporate the water using thermal energy. It is often used for degradation of organic material in the sludge and reduction of its volume, making its storage, transportation, packaging and retail easier (Levlin, 2009; IWA, 2018);
- **Incineration:** reduction to ashes. The heat generated from the sludge can be used as final product of this process, as thermal energy. This energy can be recovered for the drying process or transformed into steam for electricity production (other uses of the ashes are possible such as disposed of in landfill) (Bolin, 2009; Waterleau, 2018; Xu, 2014);
- **Anaerobic digestion:** it's a biological degradation for stabilization of organic wastes, where methane is produced as by-product, being a source of renewable energy production (Wyant et al., 2013).

Apart from this, the major current destination of dewatered sludge from WWTP is as raw material for fertilizers to be used in agriculture (Gurjar et al., 2017; Simões, 2015). The high levels of organic matter in the sludge, as well as the existence of macro and micro nutrients, essential for the adequate growth of the crops, act in the correction and/or the fertilization of the soil. If and when sludge is not adequately stabilized, when its fermentation power is not reduced, causing the production of gases and odours, or when contains high levels of undesirable compounds (e.g. heavy metals), it must be disposed of in landfills (Moço, 2012).

2.1.5.1 Anaerobic Digestion: Biogas Production

The anaerobic digestion, one of the referred techniques of sludge treatment, emerged as an alternative of great environmental and economic value. This process allows the reduction of the mass of solids by the conversion of the organic fraction into biogas. The biogas is then converted into energy and heat in cogeneration units, contributing to the economic sustainability not only of this technique, but also of the whole WWTP facility. Besides, the final solid residue reaches a higher degree of

microbiological stabilization, reducing the risk of soil contamination with pathogenic microorganisms (Simões, 2015; Tottie, 2008).

The composition of the biogas produced is variable (an example is given in Table 1), and it depends on the composition of the sludge.

Table 1 Typical composition of the biogas in a WWTP (adapted from (Hernández, 2015; Biarnes, 2018))

COMPOUND	COMPOSITION (%)
CH_4	50-80
CO_2	20-50
H_2	0-5
O_2	0-1
CO	0-1
N_2	0-3
H_2O (STEAM)	SATURATION
H_2S	0-1
SILOXANES	0-100 mg/m^3
OTHER (NH_3, HCH, ETC.)	TRACES

These characteristics are relevant to define an adequate purification system. It is important to remove impurities (such as hydrogen sulphide), and to reduce the steam, halogenated compounds and volatile compounds of silicon from biogas, in order to decrease harmful effects in the equipment (Figure 2), causing extra expenses to WWTPs (Chottier et al., 2014; Moço, 2012). In fact, the latter compounds – volatile methylsiloxanes (VMSs) - and their effect in WWTPs, are the focus of this work.

Figure 2 Acid corrosion, sulphur and silica deposits in engines after exposure to non-purified biogas (Arnold, 2009; BGS, 2018; EPA, 2002).

According to one report cited by McCarrick (2012), removal of siloxanes can save, in a 5 million-gallon-per-day wastewater treatment facility, $60,000 to $130,000 per year in operating costs. Therefore, there is a concern to purify the biogas in WWTPs.

2.2 Siloxanes

2.2.1 Description

According to IUPAC, siloxanes can be inorganic (due to their Si-O backbone) or organic compounds, since sometimes they comprise organic subunits (McNaught et al., 1997). Siloxanes

consist of silicon atoms linked by oxygen atoms. Each silicon atom bears one or several side organic chains (e.g. -CH_3), which may form cross-links and influence the properties of the polymer (Gaj et al., 2015; Greve et al., 2014). The physicochemical properties vary, depending on their molecular weight. The very low electronegativity of Si (1.8) leads to a very polarized Si−O bond with large bond energy (108 kcal/mol) (Lassen et al., 2005).

Organic siloxanes are commonly divided in three major classes: volatile methylsiloxanes (VMS), polydimethylsiloxanes (PDMS) and functionalized siloxanes (Chottier et al., 2014). In general, siloxanes have low water solubility. In fact, low molecular weight molecules are slightly soluble in water, while PDMSs are almost insoluble (Rücker et al., 2015). The most common are polydimethylsiloxanes (PDMS), with different modifications (Gaj et al., 2015). Linear low molar weight PDMS with the simple repeating unit $(CH_3)_2SiO$ are used as surface – modifying additives to achieve desired surface chemistries (Chauhan et al., 2013). PDMS can be found in a wide variety of industrial applications and consumer products, including cosmetic products and medical devices, but they are not volatile structures (Wang et al., 2009).

On other hand, VMSs can adopt two basic conformations: linear and cyclic (Figure 3). Linear structures are usually expressed as Ln, whereas cyclic ones usually follow the notation Dn, where n in both cases is the number of silicon atoms in the molecule (Rücker et al., 2015).

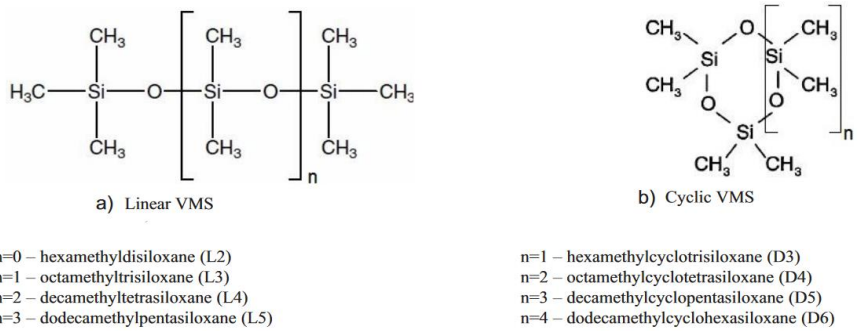


Figure 3 Primary molecular structures of (a) linear and (b) cyclic volatile methylsiloxanes (Gaj et al., 2015).

In this class of compounds, the water solubility decreases with the increase on the chain length. They are characterized by their high stability, biocompatibility, surface activity and lubricating properties (Bletsou et al., 2013). Either as single substances or as mixtures they are extensively used as carrier solvents and emollients in cosmetics and personal care product (PCP) formulations (Dudzina et al., 2014), being also incorporated in consumer products, including detergents, paper coatings and textiles (Gaj et al., 2015; Lassen et al., 2005). This work will focus in the class of volatile methylsiloxanes (VMSs). The “volatile” denomination is due to the fact that their boiling points are below 250 °C (Brebbia et al., 2011).

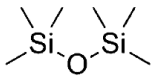
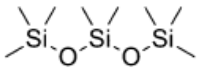
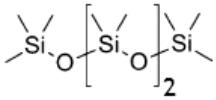
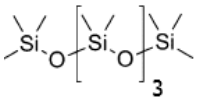
Due to their properties, that distinguish them from the already existing components, it is hard to find substitutes. In goods like soaps and leave-on products (lotions and creams), they can give the

smooth and soft feeling on the skin, combined with the sense that the product does not feel greasy after application. Furthermore, the alternatives that exist are more expensive, and none have the same solvent, emulsifier and anti-soiling agent multi-functionality (Lassen et al., 2005).

2.2.2 Linear Volatile Methylsiloxanes (lVMSs)

Linear volatile methylsiloxanes (lVMS) are mainly used as intermediates in the production of silicon polymers and, on a smaller scale, as carriers in personal care products (PCPs). Usually lVMSs represent the lowest concentrations of the total VMSs present in PCPs (Panagopoulos et al., 2018). Among this class, the lVMSs that are considered most relevant are characterized in the Table 2.

Table 2 Chemical structure and some physicochemical properties of lVMSs.

Compound CAS no. Molecular formula	Chemical structure	Molar mass[a] (g/mol)	Boiling point[b] (°C)	Log K_{ow}[c]	Water solubility[b] (mg/L, 25 °C)	Vapor pressure[c] (mmHg, 25 °C)
Hexamethylsiloxane (L2) **107-46-0** **$C_6H_{18}OSi_2$**		162.38	107	4.20	9.30×10^{-1}	31.00
Octamethyltrisiloxane (L3) **107-51-7** **$C_8H_{24}O_2Si_3$**		236.53	153	4.80	3.40×10^{-2}	3.90
Decamethyltetrasiloxane (L4) **141-62-8** **$C_{10}H_{30}O_3Si_4$**		310.69	194	5.40	6.74×10^{-3}	0.55
Dodecamethylpentasiloxane (L5) **141-63-8** **$C_{12}H_{36}O_4Si_5$**		384.84	232	6.00	3.09×10^{-4}	0.07

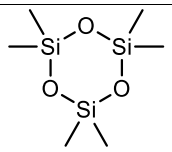
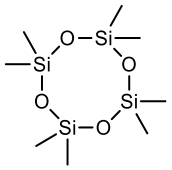
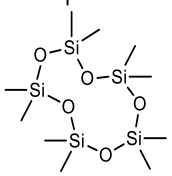
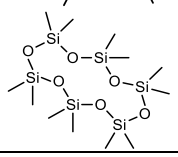
[a]Sanchis et al. (2015), [b]Schmitt (2014); [c]Kim et al. (2013).

The log K_{ow} of the linear VMSs is above 4, which proves a lipophilic nature for all of them. It is also noticeable that lipophilicity increases with the chain length, as well as with boiling points (which range between 107 °C and 230 °C).

2.2.3 Cyclic Volatile Methylsiloxanes (cVMSs)

In the cyclic siloxanes, the Si-O backbone forms a cyclic structure (a ring arrangement) with two substituents (methyl groups) attached to each silicone atom (CECBP, 2008). cVMSs are often used in the manufacture of silicones, in combination or alone in PCPs, and as carriers, lubricants and solvents in a variety of commercial applications. Due to its wide application, they tend to occur in environmental media, and also in sewage sludge. Some studies shown that D5 was the dominant siloxane in all environmental matrices sampled, except for air, where D4 dominated (Kaj et al., 2005). The most representative cVMSs are characterized in Table 3.

Table 3 Chemical structure and some physicochemical properties of cVMSs.

Compound CAS nr. Molecular formula	Chemical structure	Molar mass[a] (g/mol)	Boiling point[b] (°C)	Log K_{ow}[c]	Water solubility[b] (mg/L, 25 °C)	Vapor pressure[c] (mmHg, 25 °C)
Hexamethylcycloclotrisiloxane (D3) **541-05-9** **$C_6H_{18}O_3Si_3$**		222.46	135	4.47	1.56×10^{0}	10.00
Octamethylcyclotetrasiloxane (D4) **556-67-2** **$C_8H_{24}O_4Si_4$**		296.62	176	5.10	5.60×10^{-2}	1.30
Decamethylcyclopentasiloxane (D5) **541-02-6** **$C_{10}H_{30}O_5Si_5$**		370.77	211	5.20	1.70×10^{-2}	0.40
Dodecamethylcyclohexsiloxane (D6) **540-97-6** **D6** **$C_{12}H_{36}O_6Si_6$**		444.92	245	6.33	5.50×10^{-3}	0.02

[a]Sanchis et al. (2015), [b]Schmitt (2014); [c]Kim et al. (2013).

As well as in the lVMSs, cVMSs present log K_{ow} values higher than 4, implying a lipophilic behaviour. For the water solubility values, they decrease with the size of the ring. The boiling points ranged from 134 °C to 245 °C. D6 is the less volatile compound.

2.2.4 Environmental Concern and Ecotoxicity

It is known that the presence of VMSs in environment matrices (such as air, water, biota and soil/sediment) is not natural, and adverse toxicological effects are possible (Cortada et al., 2014; Kulkarni, 2012). Thus, the fact that cVMSs have been reported in different matrices, led to pursuit its toxicological behaviour (Rocha, 2017). Examples of matrices where they have been detected were pelagic food webs of two Norwegian lakes and in brown trout in aqueous environments receiving discharges from WWTPs (Borgå et al., 2013); bottom fish samples in marine environment in Northeast China (Hong et al., 2014); vegetation, phytoplankton and krill in Antarctic (Sanchis et al., 2015) and in pine needles in Portugal (Ratola et al., 2016). Among the cVMSs, the most reported and studied compounds are D4 and D5. This can be due to the fact that most commercial formulations, especially PCPs, have them in their composition. D4 is considered a persistent, bioaccumulative and toxic (PBT) substance and D5 is a very persistent, very bioaccumulative (vPvB) substance as agreed by the EU Member State Committee. Due to these properties, they have the potential to accumulate in the environment and cause unpredictable long-term effects, which may be difficult to reverse (ECHA, 2016). Besides, PCPs are used daily. Hence, it is necessary to infer its toxicological effect. To understand that, some studies were carried in different species:

i. D4 and D5 were tested in rats. Results showed that they did not cause adverse effects on skin or eyes and respiratory sensitization were not identified. On the other hand, sub-acute and sub-chronic toxicity studies shown that the liver and the lung of the animals are affected by D4 and D5, respectively. They also indicate that the critical effects of the siloxanes are impaired fertility (D4) and potential carcinogenic effects (uterine tumours in females). D5 was not considered genotoxic (Dekant et al., 2016; Lassen et al., 2005).

ii. Having in mind that PCPs will end-up in wastewater, it is also relevant to understand if the final effluent (produced in WWTPs) that will be discharged to the environment, will affect the receiving water bodies and the surrounding species. Therefore, in aquatic organisms, D4 and D5 levels were usually above 2000 L/kg, which meet the bioaccumulative (>2000 L/kg) or very bioaccumulative criteria (>5000 L/kg). A study showed that its bioaccumulation character is a concern, especially at lower trophic levels. D4 is very toxic to sensitive aquatic organisms, which meets the toxicity criterion of the European Commission (EC) - long-term non-observed-effect-concentration (NOEC) for marine or freshwater organisms is <10 µg/L. However, due to their low values of solubility in the aqueous media, generally, VMSs were reported as non-toxic to a great extent of the studied organisms (Wang et al., 2013).

iii. On other hand, it should be taken into account that in WWTPs these pollutants will mainly accumulate in sludge, which may be used in the agriculture after proper conditioning. For that reason, VMSs may be carry through the soil, being relevant the study of their ecotoxicity in this type of matrix. There is only one study regarding the ecotoxicity of D5 in soil. It reports that more than half of D5 was lost over the duration of the tests (i.e. 14–28 d). It is expected that a higher percentage of D5 is lost through evaporation/degradation and, depending on the relative humidity and other soil characteristics such as clay content, clay type and pH, siloxanes would not persist longer then 1–5 d in soil. Hence, the observed percent loss was less than expected, given the volatility of D5 and the fact that the test systems were not 'closed' vessels (i.e. test organisms require air exchange). The toxicity of the D5 was species and endpoint dependent. No significant adverse effects were observed for *T. pratense* or *E. andrei* tested endpoints. However, toxicity was observed for *H. vulgare* plant growth and *F. candida* survival and reproduction (Velicogna et al., 2012).

Detailed information on the conditions and concentrations of the studied cVMSs regarding the reported cases is given in Appendix 1 Ecotoxicity. In short, in the long-term, VMSs can persist and accumulate, causing adverse effects in the surrounding environment, for both sensitive and other still not evaluated species. Thus, it is important to study a way to eliminate them and preserve the ecosystems.

2.2.5 Impact of VMSs in Wastewater Treatment Plant

Although most VMSs disperse into the atmosphere, where they are eventually decomposed, some end up in wastewater streams (McCarrick, 2012). Due to their low water solubility and high sorption coefficients (they are considered more adsorptive than many organic compounds), once discharged down-the-drain, VMSs adsorb to particulate matter and settle down with sludge during the wastewater treatment (Bletsou et al., 2013). Although they do not tend to accumulate in the water phase, the absorption on the sludge is due to the extracellular polymeric substances (EPS) of the sludge flocs. Then, they end up in the biogas produced during sludge digestion, especially D4 and D5. Smaller molecules, such as D3, volatilise rapidly and are only present in wastewater in small amounts. On the other hand, larger molecules, such as D6, do not volatilise as easily as the other molecules during the sludge digestion, due to their low vapour pressure (Dewil et al., 2007). Unfortunately, when biogas contaminated with siloxanes is burned, those compounds are converted into silicon dioxide particles, which are chemically and physically similar to sand. This can cause significant internal damage to turbines and other motors (just imagine sand grinding away in your automobile's engine). The same problem occurs with biogas used for fuel cells (McCarrick, 2012). Once again, according to one report cited by McCarrick (2012), the removal of siloxanes can save, in a 5 million-gallon-per-day wastewater treatment facility, $60,000 to $130,000 per year in operating costs. Therefore, there is a concern to purify the biogas in WWTPs. There are already some techniques to remove siloxanes from biogas, to prevent equipment damages (Ruiling et al., 2017; Soreanu et al., 2011). Table A1, presented in Appendix 2 Biogas Purification Techniques, sums up these alternatives, which, nevertheless, present some disadvantages that can hinder their use in WWTP. Summing up the disadvantages, those alternatives are expensive (Arnold, 2009), and some are not efficient enough (Ruiling et al., 2017; Soreanu et al., 2011). Thus, to understand at what levels VMSs are present throughout the whole WWTP process, a good initial point would be to tackle the problem directly in the sludge, preventing them from trespassing to the biogas and, consequently, to the cogeneration engines.

2.3 Methods for the Analysis of VMSs in the Sludge

2.3.1 Extraction and Clean-up Techniques

To determine linear and cyclic VMSs in sludge samples, some extraction and clean-up techniques have been suggested, such as solid-liquid extraction (SLE), ultrasound extraction (USE), solid-phase extraction (SPE), QuEChERS and purge and trap extraction.

The basic principle of solid-liquid extraction (SLE) is the diffusion of the target analytes from the solid matrix to the highly soluble solvent chosen, due to the driving force created between the two phases (solid and liquid) (Heldman et al., 1997; Pavia, 2005). There are four important factors that can

influence the process: temperature, contact area (samples can be powdered, crushed or milled to increase the surface area of the solid particles towards the liquid phase), extraction time and type of solvent. To promote the mass transfer of the analytes to the liquid phase, agitation is also often employed. Afterwards, it is possible to recover the supernatant with the target analytes through a filtration or centrifugation of the suspension (Khan et al., 2011; Ramaswamy et al., 2013).

Another extraction method widely used in sludge extraction is the ultrasound extraction (USE). It consists in adding an appropriate solvent with affinity to the analytes present in the solid matrix. After that, the sample is submitted to the effect of ultrasound waves. During the sonication process, longitudinal waves create regions of alternating compression and rarefaction of the medium, and cavitation phenomena that promote the formation of gas bubbles. Large amounts of energy are released to the medium, promoting greater solvent penetration into the sample matrix, increasing the mass transfer of the analytes to the solvent. This technique commonly reduces working times and increases extraction yields. After sonication, the supernatant can be recovered, through a filtration or a centrifugation step (Picó, 2013).

Solid-phase extraction (SPE) is a widely use sample preparation or clean-up technique to isolate the desired analytes from a liquid phase, being often used after an extraction as an anchorage step. Basically, it refers to the non-equilibrium, exhaustive removal of chemical constituents from a flowing sample via retention on a contained solid sorbent and subsequent recovery of selected constituents by elution from the sorbent using an appropriate solvent (Poole, 2003; Welch, 2004). The three major goals of this technique are pre-concentration of the target analytes, matrix simplification achieved by the removal of unwanted analytes from the sample (clean-up) and transfer from the sample matrix to a different solvent (medium exchange) (Simpson, 2000). The most critical point is, in fact, to select a solvent that effectively extracts the target analytes (JoVE, 2018). SPE, coupled with SLE, can improve the intended phase separation, the use of expensive and breakable specialty glassware, and disposal of large quantities of organic solvents. SPE is usually more efficient and faster, yields quantitative extractions that are easy to perform, and can be automated (Brown et al., 2017). Many improvements in the SPE field such as new formats (e.g. sophisticated cartridges and discs, pipette tips and 96-well plates), new sorbents and the development of automated systems, have led to an extensive use of this technique (Hennion, 1999). SPE needs less solvent than SLE, but it is a time-consuming multi-step process, and also often requires a concentration step which may result in a loss of volatile components (Mondello et al., 2002).

In order to improve the classic SPE, a new sample preparation technique was introduced in 2003 by Anastassiades et al. (2003). This method, known as QuEChERS (an acronym for Quick, Easy, Cheap, Effective, Rugged and Safe), only requires a small amount of sample and solvent as well as a single extraction/clean-up procedure using an appropriate solvent. In the first step, the extraction solvent is

added to the sample, and salts, acids and/or buffers can be added to enhance extraction efficiency and protect sensitive analytes. The second step is a clean-up procedure based on a dispersive solid-phase extraction (d-SPE), a key improvement incorporated in the QuEChERS technique. Centrifuge tubes are prefilled with precise weights of a drying agent and SPE adsorbents to remove excess water and unwanted contaminants, respectively from the extracted samples. After agitation and centrifugation, the cleaned extracts are ready for analysis. The last step is the sample analysis. Samples may be pH-adjusted to protect sensitive analytes and/or solvent-exchanged to improve analysis by either GC-MS or LC-MS (FAO, 2018; Restek Corporation, 2017).

To extract and concentrate volatile organic compounds from liquid or solid matrices for GC analysis, a purge and trap extraction is often used. This procedure is a dynamic headspace technique that reduces matrix effects and increases sensitivity, particularly useful for concentrating VOCs that are insoluble or poorly soluble in water and have boiling points below 200 °C. The sample is purged with an inert gas of high purity at room temperature or slightly heated. Volatile analytes tend to vaporize, and the inert gas is swept to an analytical trap containing an appropriate solid sorbent, which retains and accumulates the compounds of interest. This system contains multiple beds of various sorbent materials, to trap in a single tube, a broad range of high and low molecular weight compounds, polar and nonpolar. Although lower molecular weight compounds pass through the initial adsorbent beds, they are trapped by a cascade of beds, which allows the next one to protect the last, increasing active bed, preventing compounds from being held so strongly that they cannot be desorbed quickly without decomposition. During desorption, the carrier gas goes through the trap in the reverse direction of the purge flow, so that higher molecular weight compounds never come in contact with the stronger sorbents (Sigma Aldrich, 1997; Kaj et al., 2005; Restek Corporation, 2003).

After all, to separate, identify and quantify the analytes of interest retained in the extract, a separation technique is also performed. A simple explanation is given in the next chapter, where GC-MS is the used method for this project. A brief explanation of GC-FID is also given, as literature mentions it as an option for VMSs quantification.

2.3.2 Instrumental Methodologies: GC-MS

As mentioned before, after the extraction/clean-up procedure the final extract should be analysed to quantify the target compounds. For the analysis of VMSs in sludge, gas chromatography with mass spectrometry detection (GC-MS) is usually employed (Zhang, 2014). This hyphenated technique combines the separation properties of chromatography with the detection feature of MS, to identify different substances within a sample. GC is used to separate the volatile and thermally stable analytes in a sample, whereas the MS detector (usually quadrupole or ion trap) fragments the analyte to be identified based on its mass (Kataria et al., 2011). There are many advantages to use GC-

MS, including its ability to separate complex mixtures and to quantify analytes at trace levels (Cook-Botelho et al., 2017; Vazquez-Roig et al., 2012).

The sample solution is injected into the GC inlet where it is vaporized and swept onto a chromatographic column by the carrier gas (usually helium) (Stashenko et al., 2014). The separation occurs as analytes partition in and out of the stationary phase as the carrier gas moves through the column (Scott, 2016); the time it takes a specific compound to pass through the column to a detector is called its "retention time", which can be used for identification when compared to a reference (Dhaduk et al., 2017). Once the components leave the GC column, they are ionized by the mass spectrometer using electron or chemical ionization sources. Ionized molecules are then accelerated through the instrument's mass analyser. It is here that ions are separated based on their different mass-to-charge (m/z) ratios (Bul, 2008). A higher specificity is achieved by using this detector, rather than with others (Gordon, 2013). A scheme of a GC-MS chromatograph is shown in Figure 4. More detailed information is given in Appendix 3 GC-MS.

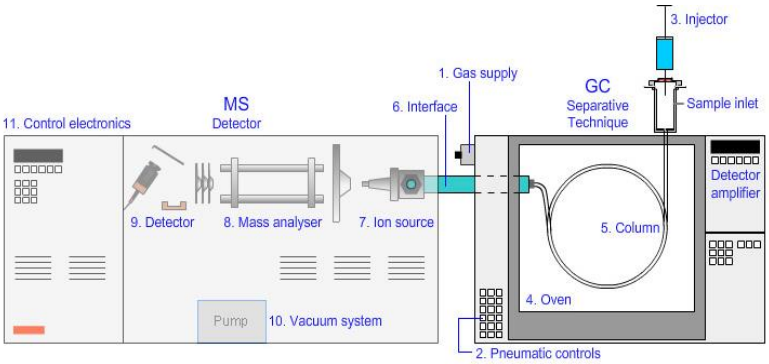


Figure 4 Scheme of a GC-MS instrument (Crawford Scientific, 2017).

Another detector reported to quantify VMSs (although to a lesser extent) is the flame ionization detector (FID). In this system, ions and free electrons are formed during the combustion of the analytes in a hydrogen flame. The charged particles produce a measurable current flow in the gap between two electrodes in the detector. This results in a recordable signal differential that is proportional to the amount of the target compounds (Holm, 1999; Zachar et al., 2018; Thomas et al., 2014). This type of detector is extremely sensitive.

3 State of the Art

To quantify and determine VMSs in sludge, most of the studies in literature used GC-MS analysis. As already seen, before quantification, it is important to extract the desired analytes from the matrix. Depending on the extraction method, the quantification can lead to better results. Through the analysis of the already existing studies, it is possible to conclude that the extraction/clean-up methods used more frequently to determine VMSs in sludge are solid-liquid extraction (SLE) and solid-phase extraction (SPE).

Through an analysis of the extraction methods, in the SLE studies, the predominant extraction solvents were: (1) hexane (Hex) (Bletsou et al., 2013; Companioni-Damas et al., 2012; Dewil et al., 2007; Oshita et al., 2014; Tavazzi et al., 2012; Wang et al., 2015) and mixtures of solvents as (2) hexane:dicloromethane (Hex:DCM) (Bletsou et al., 2013; Zhang et al., 2010) and (3) hexane:ethyl acetate (Hex:EtAc) (Bletsou et al., 2013; Li et al., 2016; Liu et al., 2014; Tavazzi et al., 2012) in different proportions, according to the matrix/sample and target compounds. The chosen solvents in the SLE technique should have a medium-low polarity, given the low polarity of the target analytes, but they must also have the ability to penetrate into the matrix pores and should not be aggressive to the chromatograph column. That is why the main choice is Hex, since it meets almost all the pre-defined properties (Dewil et al., 2007). EtAc and DCM are considered moderately polar solvents, contrary to the Hex, which is non-polar. The mixtures of solvents are useful to have an equilibrium of removal of non-polar (high molecular weight siloxanes as PDMS) with more polar analytes (low molecular weight siloxanes as VMSs) (MIT, 2012).

Applying this extraction methodology, recoveries ranging from 54±7% for L3 (Bletsou et al., 2013) to 125±15% for D4 (Zhang, 2014) were achieved.

As mentioned before, SPE was also used in several studies as a clean-up method (Companioni-Damas et al., 2012; Kaj et al., 2005; Li et al., 2016; Zhang et al., 2010). However, prior to this clean-up step, the analytes had to be extracted from the matrix, usually using the conventional SLE procedure. The sorbents used in the SPE are slightly polar in order to remove interferents and obtain a cleaner extract containing the desired analytes. In this case, recoveries between 71% for L2 (Tavazzi et al., 2012) to 95±12% for D4 (Companioni-Damas et al., 2012) were found.

Besides SLE and SPE, one study also uses an ultrasound extraction (USE), in order to achieve better extraction of the analytes in comparison to the SLE technique and in a shorter time interval (Li et al., 2016). Higher recoveries were obtained (between 74±10% and 97±5% both for L6-L16).

Summing up and analysing the recoveries obtained for the different extraction procedures, results show that they are in the same order of magnitude. From the different procedures and conditions it is also possible to verify that the lowest limits of detection (LOD: 0.002 for D4 to 0.5 µg/kg dw for L3) were achieved with a SLE-GC-MS procedure proposed by Bletsou et al. (2013). These values are slightly lower than the other studies found in literature, especially when compared to Tavazzi et al. (2012) – LODs

between 5 to 60 µg/kg dw for L2 and D6, respectively. This may be explained due to different operational conditions used in the analysis equipment.

The most investigated VMSs in all studies reported in Table 4 were the cVMSs. This may be due to the fact that they are more present in PCPs and, therefore, they are expected to be present in the sludge. The recoveries were generally higher for cVMSs (from 63% (D5) to 125% (D4) in (Zhang, 2014)), rather than the lVMSs, which ranged from 53.9% (L3) (Bletsou et al., 2013) to 95% (L5) (Companioni-Damas et al., 2012). Among cVMSs, D4 and D5, present very similar recoveries (D4: 71%-125%, D5: 63-101%). Long chain lVMSs (>L5) generally led to higher recoveries (Bletsou et al., 2013; Liu et al., 2014). For example, Bletsou et al. (2013) found that low molecular weight lVMSs had recoveries ranging from 53.9% (L3) to 72.8% (L5), while longer lVMSs (L6-L14) had higher recoveries, fluctuating between 76.4 and 93%. This may be due to the fact that they do not volatilize as easy as the lVMSs with smaller chains.

To understand at which levels VMSs are found in different places across the globe, their concentrations were determined in various WWTPs. In Athens, Greece, sludge samples were collected from a WWTP during 7 consecutive days (Bletsou et al., 2013). The concentrations observed in cVMSs were ranging from 7 µg/kg dw (mean: 9 µg/kg dw) for D3 to a maximum concentration of 17500 µg/kg dw (mean: 15100 µg/kg dw) for D5. The highest concentration registered of lVMSs was for L10, with a maximum of 12400 µg/kg dw (mean: 11300 µg/kg dw), and the lowest was for L4 with 50 µg/kg dw (mean: 56 µg/kg dw), proving that linear compounds with longer chains are present in higher levels in the samples. The linear compounds comprised 72% of the total amount of siloxanes present in the sludge.

On other hand, in the Nordic environment (Kaj et al., 2005), a purge and trap procedure was used to measure volatile organic compounds (VOCs). A thermal desorption system coupled with GC-MS with Tenax TA as the trapping agent and nitrogen as purge gas was successfully applied in different matrices (sludge, sediment, water and soil samples). In this study, the target analytes analysed were from D4 to D6 and from L2 to L5. Biologically-digested sewage sludge samples from different Nordic countries (except Iceland, where samples were from mechanical treatment only) were collected to quantify these compounds. The average concentration of cVMSs from all the analysed samples was 26000 µg/kg dw. Finland showed the highest concentration of cVMSs, 100000 µg/kg dw. In all the samples, D5 was, once again, the major contributor (mean: 11000 µg/kg dw). The lVMSs occurred in lower concentrations (110 µg/kg dw) than the cyclic analogues. The highest concentrations of lVMSs were from sludge collected in Copenhagen. In all samples the levels increased from L2 to L5, being L2 not detected maybe due to its high volatility and L5 between 24 to 46 µg/kg dw.

A different type of method was reported by Tavazzi et al. (2012), who analysed a limited number of samples (n=12). By combining a solid-liquid extraction with a clean-up step (performed with aluminium oxide), it was possible to quantify the target analytes (L2-L4 and D4-D6). cVMSs showed the highest concentrations in the sludge (<LOD for D4 to 28000 µg/kg dw for D5), with D5 being the predominant

compound. In relation to lVMSs, their concentrations varied between <LOD (L2 and L3) and 250 µg/kg dw (L4).

In other study, sludge samples were collected in the City of Loveland WWTP, Loveland and Drake Wastewater Reclamation Facility (WWRF), Fort Collins CO (both of the plants use activated sludge process to treat wastewater). Using a reverse osmosis technology (Zhang, 2014), analytes concentration were assessed, from primary sludge (PS), return activated sludge (RAS), thickened waste activated sludge (TWAS) and digested sludge (DS). In all the sludge samples, D5 was present in higher concentrations (PS: 0.007 µg/kg dw; DS: 0.0234 µg/kg dw; TWAS: 0.0314 µg/kg dw; RAS: 0.021 µg/kg dw) and D4 registered lower concentrations (PS: 0.0003 µg/kg dw; DS: 0.0004 µg/kg dw; RAS: 0.0002 µg/kg dw).

In China, 17 VMSs were determined in the dewatering anaerobic digested sludge of 42 WWTPs (Liu et al., 2014). The extraction was performed through USE and a SPE as clean-up step was used. The results show that cVMSs are, once again, the predominant compounds, with a mean concentration of 1980 µg/kg dw (ranging from <LOQ for D4 to 36000 µg/kg dw for D6), while the lVMSs produced a mean concentration of 937 µg/kg dw (ranging from <LOQ for L6 to 13000 µg/kg dw for L16). In this study, D4 was the predominant VMS (45%). This may be explained due to the lack of restrictions in the D4 use in China, contrary to the situation in Europe and North America (Liu et al., 2014). As for the linear compounds, the concentration levels increased with the Si-O chain length; for linear from L11-L16, the total concentration accounted for 84% of all the studied linear siloxanes.

Sludge samples from 6 different WWTPs with primary and secondary treatment in Spain were also studied (Companioni-Damas et al., 2012). After SLE, SPE using a silica cartridge (100 mg, 1 mL), was performed as a clean-up step. lVMSs were detected in concentrations of 4.8 (L3) to 55 µg/kg dw (L5), while the cVMSs were from 670 (D3) to 82112 µg/kg dw (D5), being the cyclic compounds, as expected, the most predominant.

Another study was done using waste activated sludge samples obtained from the full-scale WWTP of Deurne-Schijnpoort, located in Belgium (Dewil et al., 2007). The samples were collected from the secondary clarifier. Once again, using SLE it was possible to quantify the siloxanes (D4 and D5) in the samples, using a GC-FID. The lower volatility of D5 versus D4 explains why the former is only released to a minor extent, remaining at higher concentration in the sludge (253 µg/kg dw for D5 and 0.9 µg/kg dw for D4). Apart from the other analytical methods where a MS detector was used, the validation procedure of this one confirms the excellent recovery and repeatability of GC-FID.

Oshita et al. (2014) analysed two different types of sludge: (1) thickened sludge, a mixture of primary sludge thickened by gravity (TS) and excess activated sludge, thickened by centrifugation (DS) were obtained before the anaerobic digestion process at the WWTP, and (2) digested sludge, after the anaerobic digestion process. The target analytes were D4, D5 and D6, being, as expected and in accordance with most of the previous studies, D5 the predominant compound with concentrations in TS of 3.66 µg/kg dw and in DS of 0.84 µg/kg dw. D4 had lower concentrations (TS: 0.13 µg/kg dw; DS: ND).

The losses of most of the D4 and D5 in the digested sludge, compared to the thickened sludge, were consistent with the general fact that these siloxanes are the most common biogas.

Another WWTP was studied in China, which discharges into the Bohai Sea, Dalian (Wang et al., 2015). Sewage sludge samples were collected during seven consecutive days. Four cyclic and three linear siloxanes were tracked (D3 to D6 and L3 to L5). The cVMSs have dominant concentrations in the total VMSs in this study (423 for D4 to 4170 µg/kg dw for D5; 1.24 for L4 to 164 µg/kg dw for L5).

Zhang et al. (2010) determined the levels of D4 to D7 and L4 to L16 in sewage sludge sampled from WWTPs in north-eastern China using a procedure similar to Bletsou et al. (2013). In this case, the results were somewhat different from the previous studies. lVMSs showed higher concentrations (97.7 to 3310 µg/kg dw), compared with the cVMSs that ranged from 41.8 to 1420 µg/kg dw. L10 was the predominant congener with 30 to 972 µg/kg dw; concentrations increased with the chain length until L10 and then decreased forward. As for the cVMSs, concentrations of D5 and D7 were greater than those of D4 and D6. D4, D5, D6, and D7, respectively, accounted for 2.2, 11, 4.7, and 16% of the total siloxane content, while linear siloxanes (L4 to L16) accounted for 67%. This can be due to the fact that the influent may come from an industrial facility instead of a domestic one. Besides, more linear siloxanes are studied than cyclic ones.

In Harbin, China, an investigation of the occurrence and fate of 4 cyclic (D3 to D6) and 10 linear (L5 to L14) siloxanes in a WWTP was performed (Li et al., 2016). The aim was also to assess the seasonal variations of siloxanes and to establish the mass loading of target compounds. The sludge samples (n=8) were collected from the excess sludge and aerobic sludge with stainless steel jars from the WWTP in January, April, July, and October of 2012 and extracted using sonication. The results gave a concentration in the excess sludge (ES) for cyclic compounds ranging from 500 (D4) to 10900 µg/kg dw (D5) and for linear from ND (L5) to 3700 µg/kg dw (L6-L14). As for the aerobic sludge (AS), cyclic varied from 400 (D4) to 15000 µg/kg dw (D5) and linear from ND (L5 and L6-L14) to 7100 µg/kg dw (L6-L14). This suggests that different sampling locations led to different level of siloxanes in the sludge. The aerobic sludge registers higher concentrations, although the reason was not mentioned by the authors. D5 represents 30% of the total concentration of siloxanes in both matrices.

As a general overview, cVMSs are the most detected siloxanes in sludge, with a predominance of D5, except in Liu et al. (2014). The fact that cyclic VMSs are commonly present in daily commodities and PCPs, while linear VMSs are majorly used in industrial products, can explain the majority of this first compounds (Zhang et al., 2010). The extended use of D5 probably explains its predominant presence in domestic sewage. Additional sources present in the treatment (e.g. floculant with a siloxane base) can also explain higher concentrations of cVMSs.

Depending of the type of influent received in the WWTPs, the final concentrations in the sludge may vary (domestic influents traditionally have a higher impact compared to the industrial ones). Moreover, each country may have different legal restrictions about using VMSs in the products (Liu et al.,

2014). On other hand, VMSs usage also depends on the formulations of each brand, and its presence on the market, which is related with consumer options.

It is noticeable that in every study where lVMSs were analysed, L5 or linear above had the highest concentrations. This might be because the adsorption capacities of linear siloxanes increase with their Si--O chains (Liu et al., 2014). Zhang et al. (2010) also noted that the mean concentration of lVMSs increased with the chain length from L6 to L10, but it decreased from L11 to L16, still having a general higher concentration than cVMSs in the sludge. This may be related with the strong binding affinity to particulate matter and total organic carbon (TOC), favouring their partition to sludge more strongly than cVMSs (Zhang et al., 2010).

Depending on the treatment that each sludge receives and the step where the sludge is collected in the WWTP, different results are obtained. The season also changes the solubility of siloxanes. The increase of the temperature and other changes in weather may induce people to use more PCPs (sources of siloxanes) and, for instance, to take more showers, releasing more VMSs down drain. Also, rainfall can introduce a dilution factor, which can change the concentrations of siloxanes in the wastewater and, consequently, in the sludge (Li et al., 2016; Zhang, 2014).

Li et al. (2016), Oshita et al. (2014) and Zhang (2014) proved that different types of treatment in the sludge lead to different VMSs concentrations; i.e., the siloxanes levels in the digested sludge were lower than in the thickened sludge. Because sludge volume was not changed before and after the anaerobic digestion process, the reduction in siloxane concentrations in sludge can be due to the transfer of siloxanes from the sludge to the biogas during the anaerobic digestion process (Oshita et al., 2014). Also, the secondary treatment increases VMSs concentrations due to the circulation of activated sludge, which increased the mass of siloxanes in the waste activated sludge (Zhang, 2014).

Until now, studies of VMSs in WWTPs located in Portugal are scarce. Because of the occurrence and concentration levels of siloxanes in WWTPs, it is important to make a study in these facilities, to understand whether VMSs have an impact in the environment and in the WWTPs or not. The methodology used by Liu et al. (2014), that is consistent with other methods where SLE was reported, seems to be a good starting point. Therefore, it will be the used as base for the present study.

The analytical methods reported in literature for the quantification of VMSs in sludge samples are detailed in the Table 4.

Table 4 Overview of methodologies and corresponding results of VMSs in sludge.

Country	Analytes	Extraction technique	I.M.	%REC	LOD (µg/kg dw)	Concentration (µg/kg dw)	References
		ES: Excess sludge; AS: Aerobic Sludge					
China	D4	**Solid-liquid extraction** 1 g of freeze-dried sample; 3 times oscillation-extracted with 10 mL Hex:EtAc (1:1 v/v).	GC-MS	80.3±10.2	NA	ES: 500-700 (700); AS: 400-900 (600)	(Li et al., 2016)
	D5					ES: 4600-10900 (7600); AS: 8000-15000 (10500)	
	D6					ES: 1500-1800 (1700); AS: 1700-3800 (2500)	
	L5					ES: ND-300 (200); AS: ND-800 (500)	
	L6-L14					ES: 200-3700 (2100); AS: ND-7100 (2370)	
China	D3	**Solid-liquid extraction** 1 g ww: 2 mL of ACN + 0.5 mL of Hex; mixed at a room temperature for 30 min at 2500 rpm; centrifugation at 1000 rpm for 5 min. The supernatant (0.3 mL) was placed in a vial for further analysis.	GC-MS	65	0.033*	ND	(Wang et al., 2015)
	D4			78	0.06*	423-2260	
	D5			89	0.062*	732-4170	
	D6			86	0.116*	1210-3310	
	L3			76	0.113*	ND	
	L4			91	0.045*	1.27-92.9	
	L5			88	0.096*	33-164	
		Anaerobic digested sludge collected at the dewatering process:					
China	D4	**Ultrasound extraction** 0.1 g of the freeze-dried and sieved sample:10 mL of EtAc:Hex (1:1) for 15 min in an ultrasound bath; centrifugation at 3500 rpm for 10 min; repeat the extraction for 3 times; combine supernatants; evaporation through a gentle stream of N_2 until 2 mL; purification by passing through a cartridge packed with 1.0 g anhydrous Na_2SO_4; concentrated to 1 mL under a gentle stream of N_2.	GC-MS	85±3	1.3*		(Liu et al., 2014)
	D5			84±7	1.7*	<LOQ – 36000 (1980)	
	D6			82±6	1.5*		
	L3			90±9	0.5*		
	L4			89±4	0.6*		
	L5			90±6	0.7*	<LOQ – 13000 (937)	
	L6-L16			(79±10)-(97±5)	0.5-1*		
		TS: Thickened sludge; DS: Digested sludge (expressed in mg/L)					
Non-specified	D4	**Solid-liquid extraction** 50 mL of sludge sample + (unknown volume) Hex + (unknown volume) Acet + stirred at an agitation rate of 1000–1200 rpm for 4 h with a magnetic stirrer; centrifugation (3000 rpm, 5 min); Hex layer was separated and analysed; duplicates were performed.	GC-MS	NA	NA	TS: 0.13; DS: ND	(Oshita et al., 2014)
	D5					TS: 3.66; DS: 0.84	
	D6					TS: 0.26; DS: 0.12	

Values between parentheses represent the mean concentrations; NA – not available; ND – not determined; *The values marked up correspond to LOQ instead of LOD, once the former one was NA.

Table 4 Overview of methodologies and corresponding results of VMSs in sludge (Cont).

Country	Analytes	Extraction technique	I.M.	%REC	LOD (µg/kg dw)	Concentration (µg/kg dw)	References
		PS: Primary Sludge; DS: Digested Sludge; TWAS: Thicken waste activated sludge; RAS: Return activated sludge (LOD expressed in µg/L)					
USA	D4	**Direct injection** 1.5 mL of sludge into headspace vial received 1 mL of reverse osmosis water; tightly closed and 3 min mix; direct injection of the solution.	GC-MS	125±15	0.22	PS: 0.0003-0.0017 DS: 0.0004-0.0016 TWAS: 0.004-0.0018 RAS: 0.0002-0.0008	(Zhang, 2014)
	D5			63±24	0.31	PS: 0.0006-0.007 DS: 0.0006-0.0234 TWAS: 0.001-0.0314 RAS: 0.0006-0.021	
Greece	D3	**Solid-liquid extraction** 5 g ww: dry and homogenize with 25–30 g of anhydrous Na_2SO_4; add 25 mL of Hex; shaken for 1 h and centrifuge for 5 min at 5000 g; add 25 mL of Hex:DCM (1:1 v/v) + 25 mL of Hex:EtAc (1:1 v/v); repeat the procedure 2 times and combine the extracts; evaporate under a gentle stream of N_2, recover the concentrated to a microvial with Hex washes; evaporate the extract again until almost dry with 0.5 mL of Isooctane; reconstitute the extract in Hex with 500 µL.	GC-MS	86 ± 16	0.010	7-12 (9)	(Bletsou et al., 2013)
	D4			71 ± 16	0.0020	90-130 (110)	
	D5			89 ± 22	0.0025	13400-17500 (15100)	
	D6			102 ± 18	0.0050	4730-5490 (5030)	
	D7			88 ± 12	0.01	740-920 (800)	
	L3			53.9 ± 7.4	0.50	160-260 (220)	
	L4			68 ± 10	0.30	50-63 (56)	
	L5			72.8 ± 8.4	0.020	210-250 (220)	
	L6-L14			(76.4±8.7)-(93±14)	0.03-3.3	400-12400 (6220)	
			(LOD in ww)				
Spain	L3	**Solid-liquid extraction + Solid-phase extraction** 0.5 g ww: add 2 g of anhydrous Na_2SO_4 (4 ◦C for 3 h); 10 min of shaking with 3 mL of Hex and 0.2 g of activated Cu; centrifugation (3500 rpm, 10 min); cooled to 4 ◦C for 30 min; supernatant removed; repeat the extraction procedure with 3 mL of Hex following; combination of the extracts; keep at 4 ◦C; clean-up using a silica SPE cartridge (100 mg, 1 mL): previously rinsed with 10 mL of Hex; extract at 4 ◦C loaded into the SPE cartridge (ca. 4.5 mL) + elution with 1.5 mL of Hex; final extract (ca. 5.5 mL) stored at 4 ◦C; (for cVMSs a dilution of the extract (1:200, w/w) is required for quantification).	GC-MS	89±4	0.004	4.8-45 (25)	(Companioni-Damas et al., 2012)
	L4			92±5	0.01	5.5-11 (33)	
	L5			95±3	0.04	21-55 (38)	
	D3			93±13	0.11	670-4642 (2656)	
	D4			95±12	0.11	2528-15070 (8799)	
	D5			91±8	0.14	2106-82112 (42109)	
	D6			90±7	0.03	1840-11935 (6888)	
EU	L2	**Solid-liquid extraction + Solid-phase extraction** 1 g of freeze-dried sample: 20 mL ethanol/sodium acetate buffer (1:1 v/v) + 400 µL of DEA-DCC; 2.5 h of shaking + 20 mL of Hex and 1 h of shaking; centrifugation (3000 U/min, 5 min); supernatant removed + 5 mL of Hex; evaporation to 5 mL; clean-up with Al_2O_3; elution with Hex:EtAc (90:10); evaporation under a gentle stream of N_2 until 900 µL; reconstitution in 1 mL of an unknown solvent.	GC-MS	71	5	<LOD-24 (NA)	(Tavazzi et al., 2012)
	L3			86	5	<LOD-31 (NA)	
	L4			85	5	30-250 (129)	
	D4			77	30	<LOD-2200 (492)	
	D5			91	30	2100-28000 (10825)	
	D6			90	60	810-5900 (2824)	

Values between parentheses represent the mean concentrations; NA – not available; ND – not determined; *The values marked up correspond to LOQ instead of LOD, once the former one was NA.

Table 4 Overview of methodologies and corresponding results of VMSs in sludge (Cont).

Country	Analytes	Extraction technique	I.M.	%REC	LOD (µg/kg dw)	Concentration (µg/kg dw)	References
Belgium	D4 D5	**Solid-liquid extraction** 50 mL of sample + 10 mL Hex; vortex at higher speed for 10 min; centrifugation at 4400 rpm for 5 min.	GC-FID	(73.7-94.8)±6.3 (99.3-100.8)±1.36	NA	0.9-158.9 (79.9) 0.575-235.3 (127)	(Dewil et al., 2007)
Nordic Countries	D4 D5 D6 L2 L3 L4 L5	**Purge and Trap** 2 g ww: 20 mL with MilliQ water; homogenization (high frequency, Polytron); 1 mL of the slurry was weighted into the purged with a gas stream (N_2, 50 mL/min) passing through an adsorbent trap (0.25 g Tenax TA) vessel (analytes thermally desorbed); dilution of the sample to 10 mL + 0.5 mL buffer solution (2M K_2HPO_4, 0.4M HCl, 80 g Na_2EDTA $2H_2O$).	GC-MS	NA	3.9 1.9 1.5 0.04 0.04 0.04 0.04	5500-100000 (26000) 6-1060 (110)	(Kaj et al., 2005)
China	D4 D5 D6 D7 L4 L5 L6-L10 L11-L13 L14-L16	**Solid-liquid extraction + Solid-phase extraction** 1 g sludge (previously freeze dried and homogenized): 25 mL of EtAc:Hex (1:1 v/v); mix for 30 min; centrifugation at 3000 rpm for 5 min; 2 times extraction; combination of the extracts; concentration by rotary evaporation to ≈ 2 to 3 mL of the extract + 5 mL isooctane and evaporation under a gentle stream of nitrogen to ≈1 mL; clean-up by passage through a silica gel packed glass column; elution with 12 mL DCM:Hex (1:4 v/v); eluate was concentrated until an unknown volume	GC-MS	78.7±11.3	0.5* 1* 0.5* 1* 0.28* 0.86* 0.35* 0.52* 2*	41.8–103 (63.3) 168–320 (280) 87.5–569 (179) 141–1420 (474) 97.7–3310 (1744)	(Zhang et al., 2010)

Values between parentheses represent the mean concentrations; NA – not available; ND – not determined; *The values marked up correspond to LOQ instead of LOD, once the former one was NA.

4 Technical Description

4.1 Chemicals and Materials

For this study, four cVMSs (D3, D4, D5 and D6) and three lVMSs (L3, L4 and L5) were considered. Individual standards of each VMSs were purchased (purity >97%), along with tetrakis(trimethylsilyloxy)silane (M4Q), used as internal standard (IS), from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade n-hexane (Hex), dichloromethane (DCM) and ethyl acetate were acquired from VWR (Fontenay-sous-Bois, France) and used as extraction solvents. Helium (99.999%), used in the GC-MS system, and nitrogen (99.999%) for solvent evaporation, were supplied by Air Liquide (Maia, Portugal). Individual stock solutions of each siloxane, including the internal standard M4Q, were prepared in hexane at approximately 1.0 g/L. From those individual stock solutions, mix stock solutions containing all the target analytes were also prepared in hexane. A diluted M4Q individual stock solution, with a final concentration of 1.25 mg/L, was also prepared in the same solvent. Ten calibration standards in hexane, with concentrations of each analyte ranging from 5 to 1500 µg/L (internal standard concentration of 250 µg/L) were also prepared. All the solutions were stored protected from light and at -22 °C.

4.2 Sampling

Sludge samples were collected from a WWTP in Matosinhos (Portugal), with a treatment capacity of 329 138 inhabitants equivalent. The plant comprises primary and secondary wastewater treatment, and the obtained sludge is submitted to thickening (gravitational and mechanical, for primary and secondary treatment, respectively), anaerobic digestion and mechanical dewatering (Sisaqua, 2016). Seven different sampling points were selected, in order to study the occurrence of VMSs in the sludge, along the WWTP treatment. The chosen sample points were: (1) primary settler, (2) gravitational thickening, (3) biological reactor, (4) mechanical thickening, (5) mixed sludge tank (primary thicken sludge mixed with secondary thicken sludge), (6) anaerobic digestor and (7) dehydration centrifuges. Figure A3 in Appendix 4 Sampling strategy and water content, shows the sampling locations.

Eighty-one samples were planned to be collected for further analysis. From the primary settler, forty-two samples were grabbed, for seven consecutive days, from 1 am to 9 pm, at 4 hours intervals, to study possible variations during a day. Composite samples from the grabbed ones were also prepared and analysed (n=7). Grab samples were also collected from the gravitational thickening (n=8), biological reactor (n=5), mechanical thickener (n=5), mixed sludge tank (n=4), anaerobic digestor (n=5) and dehydration centrifuges (n=5). Sampling dates were defined according to the sludge retention

time and an explanation of the plan is presented in Table A2, Appendix 4 Sampling strategy and water content.

To assure enough sample for the extraction as well as a representative amount of solids from each unit process, 5 L of each grab sample were collected in the primary settler, as well as in the biological reactor and stored in polypropylene containers. 1.5 L of thickened samples, mixed sludge and digested sludge were also taken and stored in the same type of containers. Dewatered sludge was collected in polypropylene bags, always ensuring the minimum possible contamination by using siloxane-free materials. Samples were placed in a cooler and transported to the lab.

4.2.1. Preparation of the Samples

Before extraction, all samples collected, with the exception of dewatered sludge, were decanted and the solid phase of each grab sample were placed in 50-mL conical tubes and centrifuged at 4000 rpm (2760 *g*) for 10 min (Hettich, Rotofix 32 A, Germany). The sedimented phase was then recovered and placed in glass plates. Before freeze-drying these samples (VirTis freeze-dryer, SP Scientific, USA), they were frozen at -22 °C. The samples were weighted before and after freeze-drying, in order to assess the loss of water. The water content (%) in each type of sample was determined by the Equation 1, presented in Appendix 4 Sampling strategy and water content, giving approximately: 89% for primary sludge; 87% for primary thickened sludge; 93% in secondary sludge; 90% in secondary thickened sludge; 91% in mixed sludge; 92% in digested sludge and 81% in dewatered sludge.

4.3 Extraction procedure

The method was adapted from Liu et al. (2014), optimized, and validated for the determination of seven VMSs (L3-L5 and D3-D6) in the different sludge samples. Before extraction, sludge samples were freeze-dried during at least 64 h (VirTis freeze-dryer, SP Scientific, USA), milled and sieved (mesh 35). 0.5 g freeze-dried sludge were transferred into a 50 mL conical polypropylene tube and the internal standard (M4Q) was added (125 ng) and left to equilibrate for 15 min at room temperature. A mixture of 5 mL of Hex:EAc (1:1 v/v) was added to the sample, taken into an ultrasonic bath (J. P. Selecta, s.a., Spain) during 5 minutes at 420 W and centrifuged for 10 min at 4000 rpm (2760 *g*). The extract was transferred to a 12 mL amber-glass vial, and the extraction procedure was repeated. The extracts were combined and concentrated to 1-3 mL through a gentle stream of nitrogen. After that, the concentrated extract was transferred into an amber-glass microvial, with 20 µL of isooctane (keeper solvent), through successive washes with hexane, and evaporated almost until dryness under a gentle stream of nitrogen at room temperature. The final extract was then reconstituted in 500 µL of hexane, for further analysis in GC-MS.

4.4 Instrumental Analysis

The extracted sludge samples were analysed using a Varian Ion Trap GC-MS system (Walnut Creek, CA, USA). The mass spectrometer was operated in the electron ionization (EI) mode (70 eV). The separation was obtained at a constant flow of helium (1.0 mL/min), using a Low-bleed DB-5MS ultra-inert column (30 m × 0.25 mm, 0.25 µm). The oven temperature was programmed as follows: 35 °C (5 min) to 95 °C at a rate of 10 °C /min, then to 140 °C at a rate of 5 °C/min and then to 300 °C (5.5 min) at a rate of 35 °C/min - total time of analysis of 30 minutes. Injection (1 µL) was in conventional CP-1177 split/splitless adapted with a Merlin Microseal in split mode, with the split ratio of 100. Temperatures of manifold, ion trap, transfer line and injector were maintained at 50, 200, 250 and 200 °C, respectively. The filament emission current was 50 µA. For quantitative analysis of target compounds, selected ion storage (SIS) mode was applied. The main parameters are presented in Table 5.

Table 5 SIS mode parameters for detection and quantification of VMSs by GC-MS.

Segment Description		Identification and Quantification Parameters		
Time Range (min)	**Mass Ranges (m/z)**	**Target Compound**	**Retention Time (min)**	**Qualifier and Quantifier[(a)] Ions (m/z)**
0.00 - 6.50	Ionization Off	-	-	-
6.50 - 8.30	132-134, 190-192, 206-210	D3	7.52	133, 191, **207**
8.30 - 10.30	72-74, 130-134, 220-224	L3	8.97	73, 133, **221**
10.30 - 12.00	191-194, 264-268, 280-284	D4	11.28	193, 265, **281**
12.00 - 13.70	190-194, 206-210, 294-279	L4	12.76	191, **207**, 295
13.70 - 15.00	248-251, 266-270, 354-358	D5	14.56	251, **267**, 355
15.00 - 17.80	146-150, 280-284, 368-371	M4Q	15.30	147, **281**, 369
		L5	16.68	147, **281**, 369
17.80 - 19.50	324-328, 340-344, 427-431	D6	18.61	325, **341**, 430
19.50 - 30.00	Ionization Off			

[(a)]Quantifier ions in **bold**

4.5 Quality assurance/control and Waste Management

To minimize possible contamination of sludge samples during the experiment, some precautions were considered. Analysts avoided the use of lotions, perfumes, hand creams and other PCPs containing siloxanes. Powder-free nitrile gloves were constantly changed during the manipulation of samples. All glass material was rinsed with distilled water and acetone and the non-calibrated pieces were exposed to heating at 400 °C for at least 1 h. Procedural blanks were also analysed and posteriorly subtracted to the concentrations reported. Sample manipulation was performed in a chamber with controlled conditions.

The waste generated in this study was mainly organic solutions containing hexane, trace amounts of siloxanes and also residues of sludge. All residues were collected in proper labelled closed containers and stored, protected from light and ignition sources, for further treatment by the Environmental Management System of FEUP – EcoFEUP.

5 Results and Discussion

To extract and quantify VMSs in the sludge matrix, an analytical methodology was adapted and validated. Preliminary tests were performed in order to optimize and obtain more satisfactory and reproductible recovery results of the target analytes. Based on the literature, a methodology that had already reported good recovery results were chosen. The technique was optimized, using a assisted-ultrasound solid-liquid extraction, coupled with GC-MS analysis, to determine the concentration profile of siloxanes in the sludge matrix.

5.1 Preliminary Tests for the Development of the Extraction Procedure

Initially, the development of the extraction method was performed based on the methodology proposed by Bletsou et al. (2013), with minor adaptations. In brief, 5 g of wet sludge was mixture with 25-30 g of anhydrous Na_2SO_4 in a mortar (drying agent), and then it was spiked with 125 ng of M4Q and 500 ng of a mixture of VMSs (L3-L5 and D3-D6) and allowed to equilibrate for 15 min at room temperature. Hexane (25 mL) was added to the sample, homogenised in a vortex for 15 min and centrifuged for 10 min at 4000 rpm (2760 *g*). The extract was transferred to a pear-shaped flask, and the extraction was repeated twice with 25 mL of Hex:DCM (1:1 v/v) followed by 25 mL of Hex:EtAc (1:1 v/v). All the organic layers were combined and concentrated to 3-5 mL at 35 °C in a rotary evaporator. The extract was then transferred to a microvial with 20 µL of isooctane (keeper solvent) and evaporated under a gentle stream of nitrogen at room temperature and reconstituted with 500 µL of hexane. Using this procedure, the recoveries varied between 36±6% for D3 and 106±3% for D6. However, D4 and D5 were not recovered. In order to improve the present method, some changes were performed as may be seen in the following sub-sections.

5.1.1 Study of the influence of the mass sample

As expected by the literature review, high levels of VMSs were detected in the sludge samples (up to 1500 ng/g ww) analysed by the adapted methodology of Bletsou et al. (2013). Therefore, to speed up the extraction procedure, minimize the time of the evaporation (a limiting step due to the volatility of the target compounds (Rocha, 2017; Ratola et al., 2016)) and interferences, changes on the mass sample, and consequently, on the solvent volumes, were made. Thus, instead of using 5 g of sample, the mass was decreased to 0.5 g and the quantity of the drying agent and solvents were also adjusted. 5 mL of each solvent (Hex > Hex:DCM (1:1 v/v) > Hex:EtAc (1:1 v/v)) was used instead (enough volume to cover up all the sample) and the amount of Na_2SO_4 reduced to 5 g. The time of vortex was also reduced to 5 min, hoping that this was sufficient to homogenise and extract this smaller amount

of sample. Due to the use of less volume of extraction solvent, the volume reduction was only performed under a gentle stream of nitrogen at room temperature.

The results led to an average recovery of 67±12%, but once again, the recovery of some compounds (namely, D5 and D6) was not possible. Therefore, further tests were necessary.

5.1.2 Study of the type of extraction solvent

Maintaining the same procedure, and in order to observe the influence of the extraction solvents, the steps with Hex and Hex:DCM (1:1 v/v) were substituted by a mixture of Hex:EtAc (1:1 v/v). In fact, Bletsou et al. (2013) analysed a wide range of compounds (L4-L14 and D3-D7), with very distinct properties. For that reason, they opted to use different solvents sequentially, with increasing polarity. The less polar compounds (long chain) may be better extracted with Hex, while the lowest molecular weight VMSs with a more polar mixture of solvents (Hex:EtAc).

In this test, the recoveries were very variable for all the compounds, with high relative standard deviation for the replicates (n=3). The recoveries varied between 12±14% for L3 and 445±28% for D6 (average recovery: 167±32%), showing no reproducibility in some cases, possibly due to matrix effects. This may be explained by the poor homogenization of the sludge samples and the difficulty of removing all the water content from the sample with Na_2SO_4. Therefore, the next step was to study the homogenisation process.

5.1.3 Study of the sample homogenisation process

After the literature review, it was found that a freeze-drying step was generally applied as a sample preparation step, reducing the water content (Tavazzi et al., 2012; Li et al., 2016; Liu et al., 2014; Zhang et al., 2010). Therefore, in this work the freeze-dry procedure was also tested. Firstly, preliminary tests were performed in order to define the freeze-drying time. The initial sample mass was measured and then, different sample weightings were carried out along the freeze-dry process, until a constant weight was obtained. This was defined as the time required for the freeze-drying procedure (64 h).

After the freeze-drying process the samples were extracted maintaining all the conditions mentioned before (3x 5 mL Hex:EtAc (1:1 v/v), 5 min vortex). In these assays, all target compounds were recovered (average recovery: 126±32%), but the standard deviations were still high and for some compounds (mainly D4 and D5) a matrix effect was still verified. This may be explained by the presence of flocs and particles of different diameter in the freeze-dried matrix, which contribute to the difficulty of collecting a homogenised and representative sample.

To overcome this problem, authors decided to sieve the samples after the freeze-drying process. A stainless-steel sieve, with a pore size of 500 µm (mesh 35) was used, as well as a pestle that helped

to break up very carefully the sample. A more homogenised sample was obtained without flocs and large sludge particles, as well as other materials usually retained in the matrix (e.g. hair, stones and sticks). This procedure clearly improved the extraction and, as expected, lower standard deviations were achieved, except for D3 and D5, as proven in Figure 5 (average recoveries of 82±12%). Therefore, the following tests were performed freeze-drying and sieving the samples.

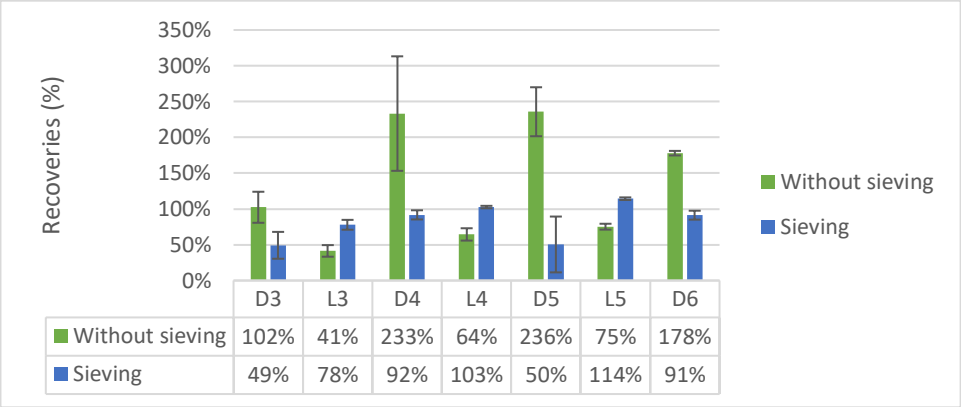


Figure 5 Recoveries (%) of the target analytes obtained after freeze-drying the sludge and after both freeze-drying and sieving the sludge (0.5 g of freeze-dried sludge, 3-fold 5 mL Hex:EtAc (1:1 v/v) , 5 min vortex). The error bars represent the standard deviations (n=3).

5.1.4 Study of the extraction mode

After these tests, the authors decided to check the influence of the extraction mode employed – vortex *vs.* ultrasound extraction (USE). Promising results using USE have already been reported by Liu et al. (2014) for the extraction of VMSs from sludge. In fact, USE could improve the penetration of the solvent in the matrix, allowing better efficiencies for the same extraction time. The comparison of recoveries for both methodologies are shown in Figure 6.

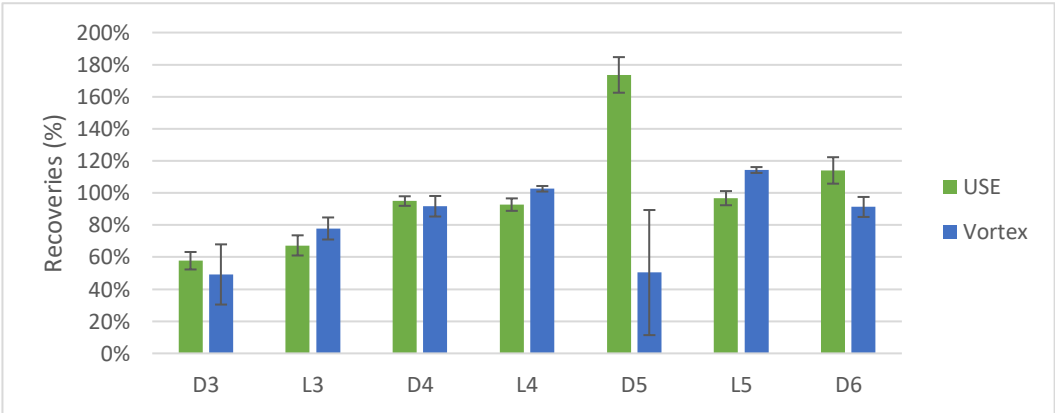


Figure 6 Recoveries (%) for the studied VMSs for both USE and vortex techniques (0.5 g of freeze-dried and sieved sludge, 3-fold 5 mL Hex:EtAc (1:1 v/v), 15 min extraction). The error bars represent the standard deviations (n=3).

USE improved the overall recoveries compared with the vortex usage, with a mean recovery of 100±6%. In general, the recoveries increased and the standard deviations were lower, especially for the critic target analytes (cVMSs). Taking into account the exposed advantage, and since this is a "user-independent" technique, the USE was used in further studies.

5.1.5 Study of the extraction repetition

In order to obtain an optimized procedure, the effect of the repetition of the extraction procedure was tested. Thus, the previous results were compared with the ones obtained by repeating the solid-liquid extraction procedure 2-fold and only 1-fold, with the same solvent mixture (Hex:EtAc 1:1 (v/v)), all in the same conditions as mentioned before. The results are presented in Figure 7.

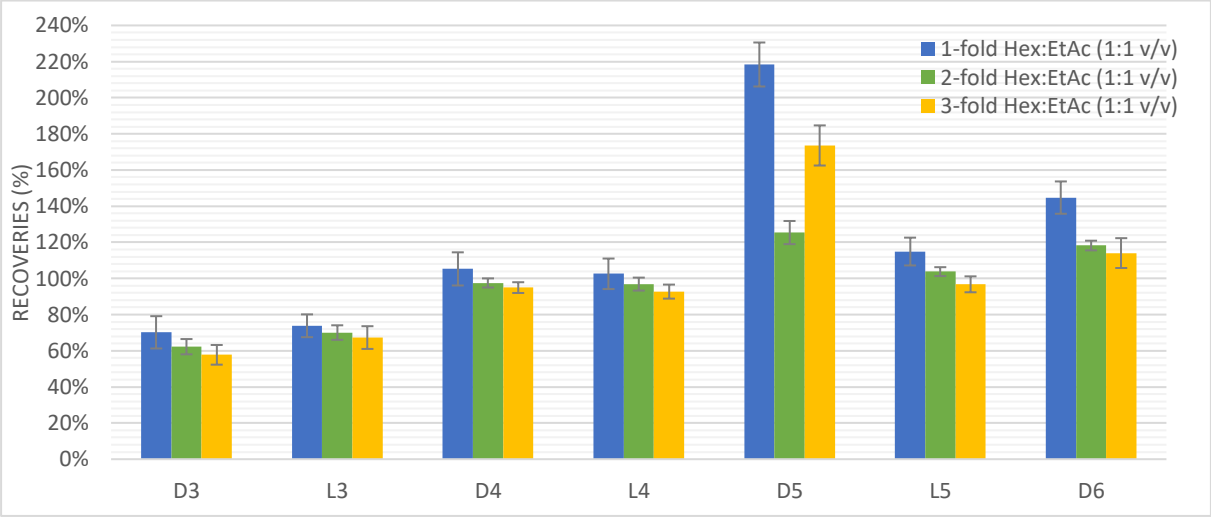


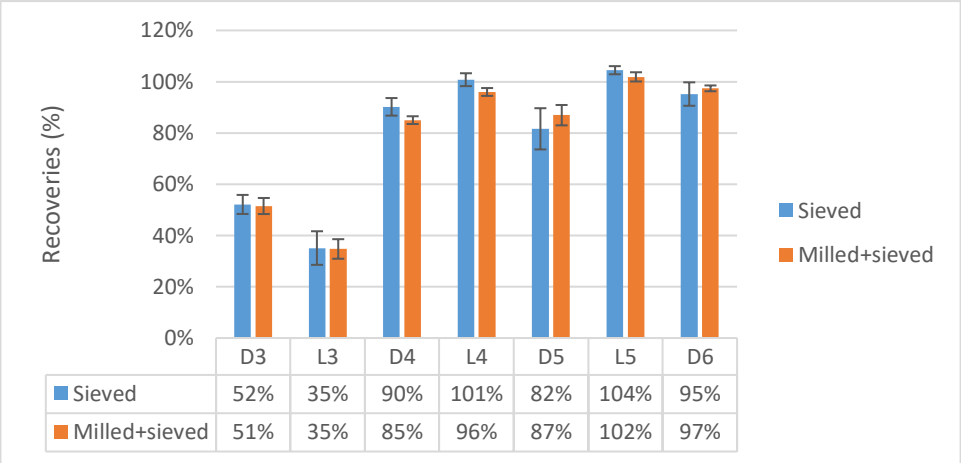
Figure 7 Recoveries (%) for the studied VMSs, testing the repetition of the extraction procedure (0.5 g of freeze-dried and sieved sludge; 5 mL Hex:EtAc (1:1 v/v), 5 min USE). The error bars represent the standard deviations (n=3).

Although it seems that 1-fold extraction with Hex:EtAc was efficient enough to recover all the analytes, the standard deviations were generally high. The recoveries ranged from 70±9% for D3 to 218±12% for D5, with an average recovery of 119±9%. The 2-fold extraction procedure was also efficient and conducted to interesting recovery values (from 62±4% for D3 to 125±6% for D5, with a mean recovery of 96±4%). In fact, the results for each target analyte are very similar, with the exception of D5, which presents better recoveries for the 2-fold extraction.

Results shown that the differences between the conditions is not statistically significant. Therefore, the 2-fold extraction procedure was preferred due to the less variability obtained for the replicates compared to the 1-fold extraction methodology and also due to solvent savings and the decrease of the evaporation time, decreasing the likelihood of losing the more volatile analytes, compared to the 3-fold extraction methodology.

5.1.6 Sample processing

Due to the high number of samples collected from the WWTP, the sample sieving process used so far was too time consuming and, for that reason, a susceptible step to risks of external contamination of the sample. Therefore, the authors opted to test the sample grinding through a mill, followed by sieving. The main recoveries are presented in Figure 8.



	D3	L3	D4	L4	D5	L5	D6
Sieved	52%	35%	90%	101%	82%	104%	95%
Milled+sieved	51%	35%	85%	96%	87%	102%	97%

Figure 8 Recoveries (%) for the studied VMSs, for different sample processing (0.5 g of freeze-dried sludge; 2-fold 5 mL Hex:EtAc (1:1 v/v), 5 min USE). The error bars represent the standard deviations (n=3).

As a first approach, it is possible to infer from the Figure above that recoveries are similar between the two ways of processing the samples. To be more rigorous, and to test the significance of the difference between the two treatments distribution, the two-tailed two-sample *t*-test was performed (Thomas et al., 2015; Jekel, 2007) and the results are shown in Appendix 5 Significance test. The level of alpha was set at p=0.05 (Jekel, 2007). The results from Table A4-A10 shown that for each siloxane, the differences between the two tested methodologies are not statistical significant (p>0.05), excluding L4, whose p-value is very close to 0.05. Therefore, in general, p- values in this two-tailed *t*-test are higher than 0.05, proving that both methodologies are similar and differences between recoveries are negligible.

5.2 Method Validation

Gathering all the established conditions, a method was implemented to detect and quantify 7 VMSs (L3-L5 and D3-D6) in the sludge samples. Validation tests were made to determine statistical parameters as linearity ranges, limits of detection and quantification, precision and accuracy.

5.2.1 Quantification Parameters

Calibration curves were plotted by direct injection of 10 calibration standards in hexane, containing all VMSs at concentrations ranging from 5 to 1500 µg/L, within a 250 µg/L concentration of IS (M4Q) (Appendix 6 Method Validation, Figure A4-A10). These curves (forced to go to the origin) were constructed correlating the quotient between the injected analyte mass and the injected IS mass, $(m_{an}/m_{IS})_{inj}$ with the response factors (RF) - the quotient between the area of the analyte and the area of the IS (Appendix 6 Method Validation, Table A11) (Alves, 2015).

To evaluate if the calibration curves are suitable to be used, quality control laboratories usually admit the following criteria: (i) the correlation factor has to be higher than 0.995 (r>0.995) and (ii) the relative standard deviation of the slope has to be less than 5% ($s_a/a \times 100 < 5\%$) (Alves, 2015). These

results are shown in the Table A12, in Appendix 6 Method Validation. The linearity responses met the criterion (i) for all the compounds (ranging from 0.996 for D3, L4 and D6 to 0.998 for L3 and L5), as well as criterion (ii). Equations to estimate the mentioned parameters are shown in Appendix 6 Method Validation, Equations 2 and 3.

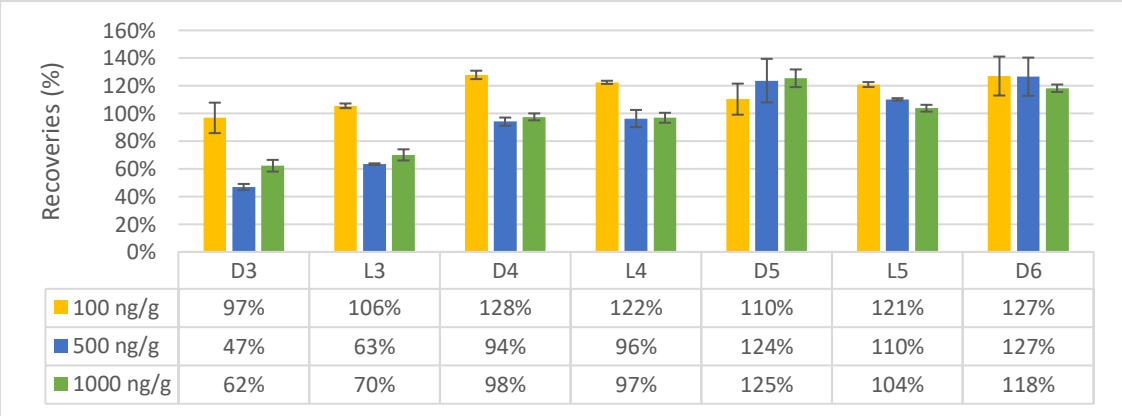
Based on the signal-to-noise ratios (S/N) of the target analytes, it was also possible to determine for each VMS the limit of detection (LOD), where a S/N of 3 was used, and the limit of quantification (LOQ), using S/N of 10. Hence, Table 6 shows the obtained LODs and LOQs for each studied VMS. LODs were similar to the ones reported by Bletsou et al. (2013) and Zhang (2014)and lower than those found by Kaj et al. (2005) and Tavazzi et al. (2012).

Table 6 Linearity range (µg/L), limit of detection (LOD) and limit of quantification (LOQ) (ng/g dw) for each VMS.

		LOD *S/N=3*	LOQ *S/N=10*
Compound	**Linearity Range (µg/L)**	**ng/g dw**	**ng/g dw**
D3	5-1500	0.11	0.37
L3		0.47	1.60
D4		0.19	0.62
L4		0.25	0.83
D5		0.0004	0.001
L5		0.18	0.60
D6		0.09	0.31

5.2.2. Accuracy and Precision

Accuracy is a measure of the nearness of the obtained analytical result to the expected value. In this study, the accuracy was assessed by recovery tests, performed using spiked samples at three different concentrations of the target compounds (100, 500 and 1000 ng/g dw) (Figure 9).



	D3	L3	D4	L4	D5	L5	D6
100 ng/g	97%	106%	128%	122%	110%	121%	127%
500 ng/g	47%	63%	94%	96%	124%	110%	127%
1000 ng/g	62%	70%	98%	97%	125%	104%	118%

Figure 9 VMSs recovery assays in the sludge samples at different spiked concentration (100, 500 and 1000 ng/g dw). The error bars represent the standard deviations (n=3).

For each VMS, the recoveries ranged from 47±2% for D3 to 128±3% for D4, with a mean recovery for all the VMSs of 102±5%, considering a mean of the different spiked concentrations. These recoveries were similar to those found in the literature (Table 4). Lower recoveries were found for D3 and L3, probably due to volatilization losses (compounds with the lower boiling points).

On the other hand, precision is a measure of the proximity between results for the same sample. Thus, repeatability (intraday precision) was assessed by the relative standard deviation (RSD) of three repeated extractions of samples spiked at three different concentrations (100, 500 and 1000 ng/g dw), while interday precision was determined by the RSD of extractions performed in three different days, at the above-mentioned spike levels. Results are shown in Table 7.

Table 7 Intraday and interday precision for each studied VMSs, at different spike levels.

	1000 ng/g dw		500 ng/g dw		100 ng/g dw	
Compound	**Intraday**	**Interday**	**Intraday**	**Interday**	**Intraday**	**Interday**
D3	4%	11%	14%	27%	12%	30%
L3	4%	8%	7%	11%	3%	22%
D4	3%	11%	10%	5%	12%	20%
L4	4%	9%	6%	4%	5%	29%
D5	6%	14%	15%	27%	8%	6%
L5	2%	9%	4%	3%	2%	26%
D6	3%	9%	9%	18%	8%	22%

The intraday precision led to relative standard deviations below 15%, which may be considered acceptable taking into account the employed method, the working concentrations and the type of matrix. As expected, the interday precision values are slightly higher (3-30%), namely for the lowest spiking levels. In fact, samples spiked with lower concentration of VMSs are more susceptible to possible cross-contaminations and losses during the extraction procedure (namely volatilization).

Thus, taking into account the type of matrix (sludge), the results obtained show that the applied methodology led to satisfactory precision values for all compounds analysed.

Chromatograms showing the differences between a standard solution of 500 µg/L of a mix solution (A), of a sludge sample spiked with the 500 µg/L of a mix solution (B) and of a sludge sample (C), all with an IS of 250 µg/L, are shown in Figure 10.

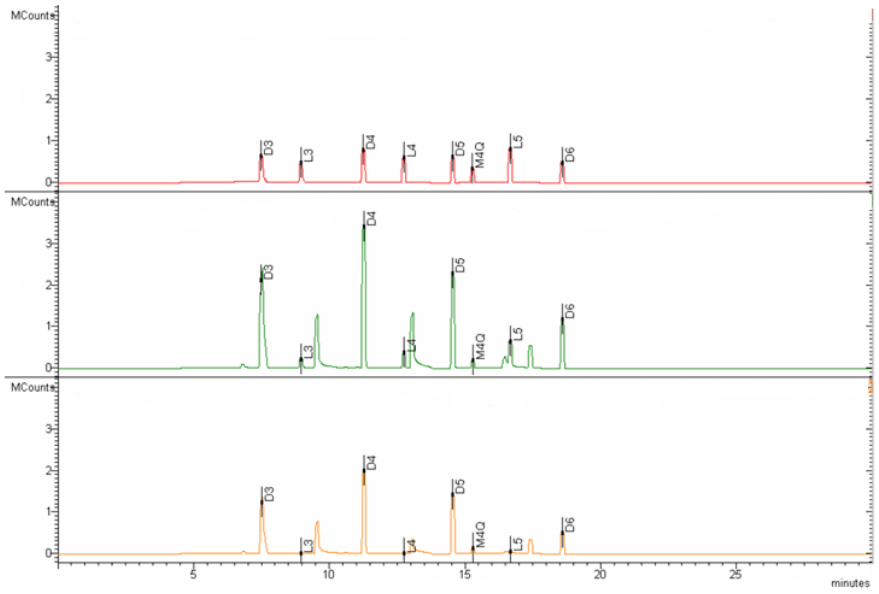


Figure 10 Chromatogram in SIS mode of: (A) a 500 µg/L mix standard solution of VMSs prepared in hexane; (B) an extracted sludge sample spiked with a 500 µg/L mix standard solution of VMSs; (C) an extracted sludge sample.

5.3. Analysis of VMSs in Sludge from Matosinhos WWTP

After validation of the proposed methodology, the sludge samples collected in the Matosinhos WWTP were extracted and VMSs were quantified (ng/g dw) according to the established procedure.

Due to technical difficulties in WWTP, it was not possible to collect all the planned samples. For example, from the primary settler (19th of May of 2018 at 9 am and 13 pm, 20th of May of 2018 at 1 am and 5 am and 21st of May of 2018 at 21 pm), gravitational thickener (20th of May of 2018 at 3 am) and mechanical thickener (21st of May of 2018 at 10:05 am).

5.3.1. Primary treatment

5.3.1.1 Primary Settler

To better understand VMSs behaviour and its variation profile in the sludge from the primary settler (SRT = 70 min), samples were collected during a week, every 4 hours, from Tuesday starting at 9 am (15th of May of 2018) to Tuesday (22nd of May of 2018), ending at 5 am (n=37). After the proper treatment and preparation, the sludge samples were extracted and the VMSs concentration profile was plotted (Figure 11; Table A13, in Appendix 7 Sample Analysis). Analysing Figure 11, it is possible to infer that the results of the first two days of sampling (Tuesday and Wednesday) did not show significant variations (total concentrations of VMSs of 1174 and 1008 ng/g dw, respectively). However, the lowest concentrations were on Tuesday at 5 pm (984 ng/g dw) and on Wednesday at 9 pm (461 ng/g dw).

An increase on the mean concentrations begin to reveal on Thursday. At 5 am and 1 pm the concentration values duplicate, showing a VMSs total concentration of 2285 and 2415 ng/g dw, respectively, against 9 pm, where the concentrations decreased sharply to 648 ng/g dw. On Friday at 5 am, a concentration peak might suggest an abnormal discharge, showing the highest concentration of the total VMSs so far of 6036 ng/g dw. Therefore, the mean concentration of this day duplicates compared with the first days (2145 ng/g dw). If we exclude this abnormal value, the average concentration would be 1367 ng/g dw. This value is similar with the average values obtained in the other days. Once again, the lowest concentration was registered at 9 pm (565 ng/g dw).

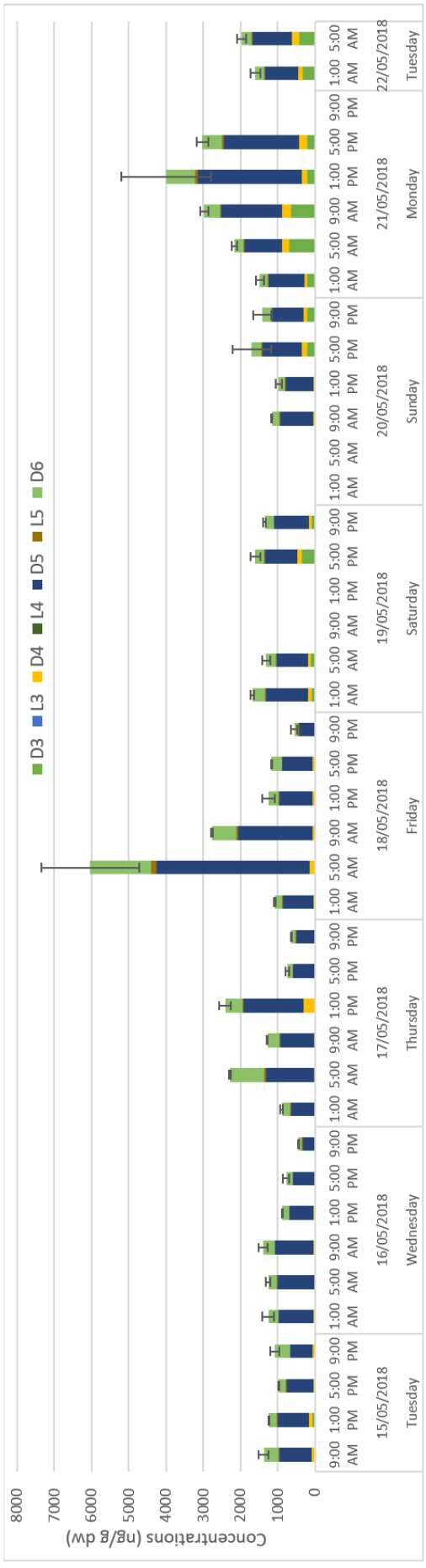


Figure 11 Daily concentration profile of VMSs (ng/g dw) in the sludge from the primary settler during a week. Error bars correspond to standard deviations (n=2).

On Saturday, the concentrations ranged from 1316 ng/g dw (5 am) to 1684 ng/g dw (1 am), being the total mean concentration of 1490 ng/g dw – a stabilization of the values is clear, with no discrepancies between the analysed hours. On Sunday, the behaviour was similar, with concentrations ranging from 983 (1 pm) to 1708 ng/g dw (5 pm), with a total mean concentration of 1319 ng/g dw. On Monday, the concentrations increased again, now showing a total mean VMSs concentration of 2733 ng/g dw, with the highest values at 1 pm (4005 ng/g dw), followed by 5 pm (3029 ng/g dw) and 9 am (2981 ng/g dw). The lowest concentration was at 1 am (1490 ng/g dw). Summing up, highest concentrations were registered on Monday. On other hand, Wednesday had the lowest concentrations. Although the variation of VMSs concentrations through some days were not significant, it is possible to conclude that, in general at 9 pm and 1 am were observed the lowest concentrations. The highest levels were recorded at 5 am and 1 pm. This behaviour suggests that from 5 am (which represents the peak of concentrations) to 9 pm (the hour where the concentrations are the lower) the values decrease, being more or less persistent during the other hours. At 1 am and 5 am they increased again.

Due to the large extension of the sewage network connected to the Matosinhos WWTP (it receives sewage from Matosinhos to Vila do Conde – distance of about 19 km), it is not easy to establish a pattern between the results obtained and the population habits. In fact, it was not possible to estimate the time of a domestic and/or industrial discharge in the sewage system occurs, based on the sampling time. It is also clear that higher concentrations were achieved for cVMSs. Among cVMSs, D5 was the major contribution for the total amount of VMSs concentration (66%), followed by D6 (20%). It is also noticeable that D3 concentrations, that were normally below detection limit, increased on Saturday until Tuesday (means: 164-380 ng/g dw). D4 was the cyclic VMSs with the lowest concentrations, varying from 15 to 317 ng/g dw. For lVMSs, variations were not significantly different among days/hours. However, L5 was the most frequently detected lVMS (8 to 153 ng/g dw) and L3 had never been detected in these samples.

Composite primary sludge samples

With the concentrations obtained every 4 hours, in each day, an average concentration of each compound per day was estimated and results are shown on Table A14, Appendix 7 Samples analysis. In order to understand if the calculations match the reality, 24 h-composite sludge samples were prepared in the laboratory by adding equal masses of the grab freeze-dried, milled and sieved sludge samples (from 5 am of that day to 1 am of the day after). Before extraction, the composite samples were homogenized. Results are shown in Table A15, Appendix 7 Samples analysis.

These composite samples were purposely made to compare with the gravitational thickener samples, which reflect the discharge of the primary settler, receiving the sludge from 5 am to 1 am of

the following day). Analyses of these composite samples revealed that concentrations of VMSs are similar to those estimated using the mean daily value for VMSs in the primary settler.

5.3.1.2 Gravitational Thickener

The gravitational thickener receives along the day the sludge that is being discharge from the primary settler (SRT = 24 h). Thus, to obtain a reflection of the sludge samples taken from 9 am of 15th of May to 1 am of 16th of May of 2018, and to compare concentrations between different sludge treatments, grab samples from the gravitational thickener were also taken from day 16th of May to 22nd of May of 2018, at 3 am (gravitational thickener discharges from 1 am to 4 am). These grab samples are, therefore, 24 h-composite sludge samples. Table A16 (Appendix 7 Samples analysis) presents the concentrations of the studied VMSs in the sludge samples collected each day in the gravitational thickener, and the plotted bar graph is shown in Figure 12.

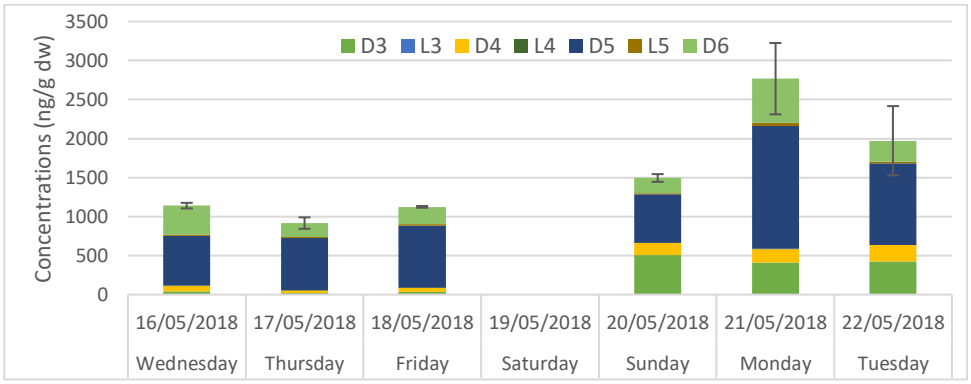


Figure 12 Concentrations of VMSs in the primary thickened sludge (ng/g dw) during a week. Error bars correspond to standard deviations (n=2).

Similar concentrations were found between samples collected from Wednesday to Friday. In the rest of the week, there was an increase in VMSs concentration, obtaining a maximum on Monday. Higher concentrations were expected on Tuesday, reflecting the increase in VMSs concentration on Monday in the primary settler. However, this was not verified, perhaps because in the primary settler, the 5 am sample on Sunday was not collected and therefore the results concerning this stage of treatment did not take into account a period of time, in which the concentrations of VMSs are usually higher, as discussed above.

Once again, the cyclic compounds are the major contributors. D5 was detected in higher concentrations (ranging from 623 to 1570 ng/g dw), followed by D6 (varied from 180 to 569 ng/g dw). D4 was the lowest of the cVMSs (ranged from 30 to 207 ng/g dw). Although lVMSs were detected in low concentrations, L5 was the predominant compound (ranging from 13 to 40 ng/g dw). L3 was not detected. By comparing the sludge that left the last unit (primary settler) with the corresponding one in this unit (gravitational thickened sludge), it is found that in general all concentrations have slightly decreased. This can mean that part of the siloxanes volatilized during the treatment.

5.3.2. Secondary treatment

5.3.2.1 Biological Reactor

Grab sludge samples were collected between 16th of May and 13th of June of 2018, at 10 am (SRT = 11 days). A sample collection on the 22nd of June was schedule, but due to time constraints, it was not possible to include it in this work. Sampling was planned in order to study the behaviour of VMSs in the sludge at this stage of treatment, including collections at times shorter than the solid retention time estimated. Results (Figure 13 and Table A17, Appendix 7 Sample Analysis) show that VMSs concentrations are higher in this unit, compared with the primary treatment. Other authors also reported higher concentrations of VMSs in the sludge from the biological reactor. Due to the high solid retention times in this unit and the characteristics of this sludge (carbon content, pH, ionic strength and the presence of complexing agents), VMSs are prone to sorb in the sludge (Bletsou et al., 2013).

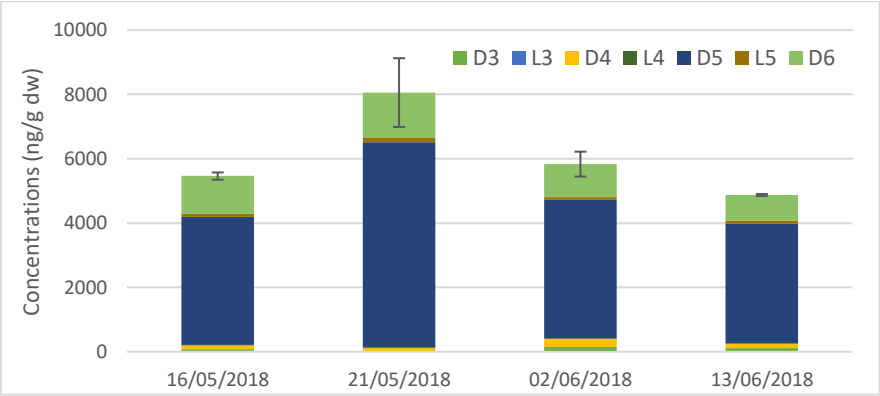


Figure 13 Concentrations of VMSs in the sludge (ng/g dw) taken from the biological reactor. Error bars correspond to standard deviations (n=2).

Through the analysis of Figure 13, it is possible to verify that from 16th to 21st of May concentrations increased (mean concentrations varied from 5463 to 8059 ng/g dw). This may mean that the sludge collected on the 16th May is in the same cycle as the one collected on 21st May. Hence, authors will be analysing the same sample, but with higher contact time with wastewater. Thus, VMSs removal from the wastewater occurs due to their sorption on the sludge particles (Zhang et al., 2010). On the 2nd of June, concentrations decrease (from 8059 to 5835 ng/g dw), which may indicate that a new treatment cycle began (Δt > SRT). Comparing the results from 16th of May, 2nd and 13th of June, it is found that the levels obtained in different cycles are not significantly different.

Among VMSs, cVMSs showed again the highest concentrations. D5 was the compound with the highest levels, ranging from 3720 to 6384 ng/g dw, followed by D6 (801 to 1401 ng/g dw). D3 was the cVMS detected at lower concentrations (nd to 149 ng/g dw). Once more, L5 presented the highest concentration from lVMSs (87 to 141 ng/g dw).

5.3.2.2 Mechanical Thickener

To evaluate the thickening process of the secondary sludge, samples were collected at the same time as those collected in the previous unit, but with a delay of 5 minutes (SRT=5 min). The concentrations are shown in Table A18 Appendix 7 Sample Analysis.

Figure 14 shows the variations between samples from the biological reactor and mechanical thickener, collected in the same day.

In the mechanical thickening, part of the water is removed from the sludge. This means that VMSs should be more diluted in the biological reactor than in the mechanical thickener (considering the results in wet weight). However, all the results are presented in dry weight (dw). Therefore, similar concentrations are expected to be found in these two processes. In fact, overall concentrations were similar.

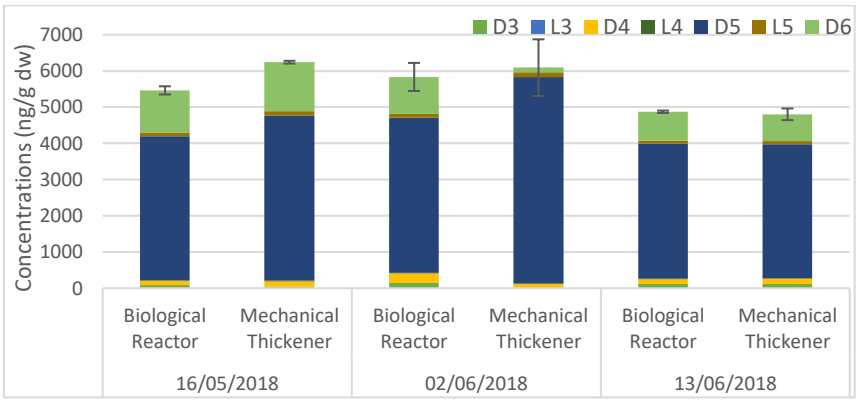


Figure 14 Comparison of the concentrations of the studied VMSs in the sludge (ng/g dw) collected from the biological reactor and from the mechanical thickener. Error bars correspond to standard deviations (n=2).

Another fact observed in Figure 14 is that D5 concentration increased slightly in the sludge, consequently, the concentration of cVMSs (where concentrations were ranging from 3719.9 (13th of June) to 6838.7 ng/g dw (21st of May) in the sludge from the biological reactor increasing from 3696.3 (13th of June) to 5702.3 ng/g dw (2nd of June) in the mechanical thickener). This can be explained by the fact that the cVMS with larger ring (D6) may undergo degradation reactions, converting to D5 (slight decrease in D6 levels in the thickened samples). D3, since it is the most volatile VMS, may also volatilize during the thickening process (sludge from the biological reactor: 90 - 149 ng/g dw; mechanical thickened sludge: nd - 122 ng/g dw). lVMSs concentrations in the sludge remained almost the same during this process.

5.3.3 Mixed sludge tank

The mixed sludge tank (SRT=8.5 h) receives the gravitational thickened primary sludge from the mechanical thickened biologic sludge. To establish a concentration profile in the mixed sludge tank, sludge samples were taken at 12:30 pm on 16th, 17th, 18th of May and on 12th of June. Results are shown

in Figure 15, and concentrations are summarized in Table A19, in Appendix 7 Sample Analysis.

As it can be seen in Figure A3 from Appendix 4 Sampling strategy and water content, this tank receives sludge with different ages, therefore, a direct comparison with all the results previously presented is not possible.

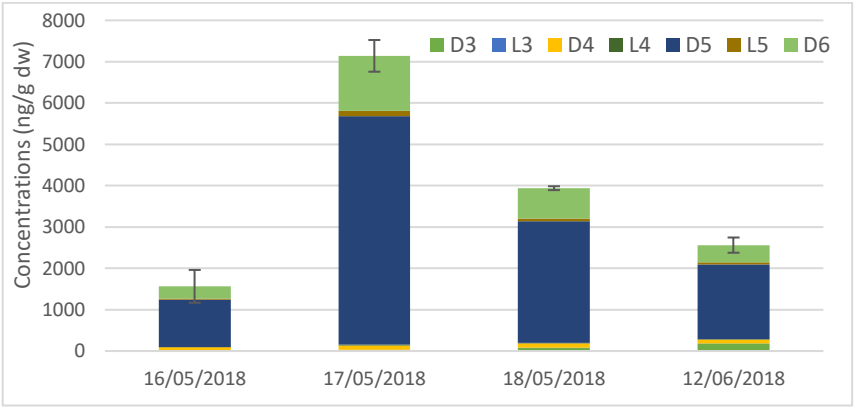


Figure 15 Concentrations of target analytes (ng/g dw) from the mixed sludge tank. Error bars correspond to standard deviations (n=2).

In the 16th of May, the overall VMSs concentration for the primary thickened sludge was 1141 ng/g dw and for the secondary thickened sludge were 62412 ng/g dw. The total VMSs concentration on 16th of May in the mixed sludge tank was 1565 ng/g dw. However, it is important to take into account that the mass flowrate is different for each entrance (71% of the total flowrate corresponds to the primary thickener and 29% to the mechanical thickener). Concentrations were higher in the 17th of May, which means that higher concentrations from the biological reactor on that day were achieved, compared with the other days. This can be assumed, since the other dates on the gravitational thickener shown that the 17th of May was the date were the lowest concentration was achieved. It is also perceptible that, once again, cyclic siloxanes have higher concentrations than linear ones, being D5 the predominant (varying from 1147 to 5520 ng/g dw), followed by D6 (296 to 1329 ng/g dw). The lowest concentration was obtainable from D3, which can mean that it may have volatilized (18 - 82 ng/g dw).

5.3.4 Anaerobic Digestion

5.3.4.1 Digester

The mixed thickened sludge is then fed to an anaerobic digester to be stabilized and this process functions as a semi-batch step, in which the sludge is fed for about 16 days (SRT = 16 days) and only at that time the stabilized sludge is discharged and conducted to a centrifuge. To understand the concentration profile inside the digester, samples were collected at time intervals of about 6 - 7 days. Table A20, in Appendix 7 Sample Analysis and Figure 16 shows the main concentration results.

It is known that the concentration inside of the digester decrease with time, since anaerobic

reactions take place, promoting biogas formation. It was already mentioned by other authors (Zhang, 2014; Oshita et al., 2014) that, when the biogas is being produced, the volatilization of siloxanes also occurs. This is due to an increase of the temperature, established to promote the anaerobic treatment.

Besides, due to the breakdown of EPS, most of the siloxanes are released from the sludge and end-up to be part of the biogas composition (Ahn et al., 2014).

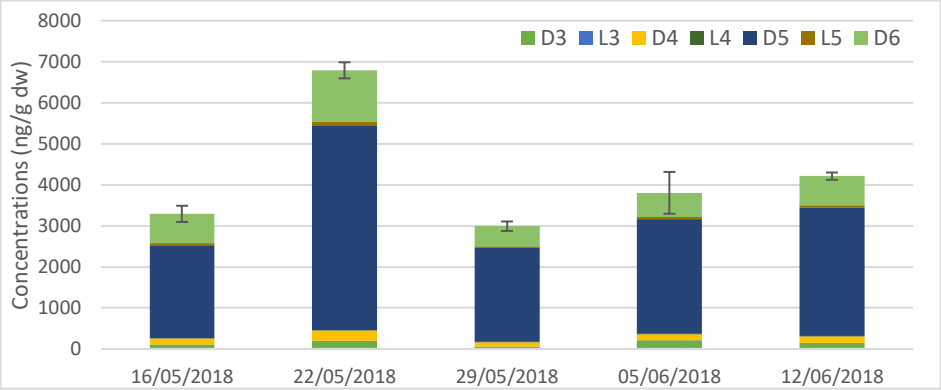


Figure 16 Concentrations of the target VMSs (ng/g dw) in the sludge collected from the digestor. Error bars correspond to standard deviations (n=2).

From 16th to the 22nd of May, the concentrations clearly increase (from 3292 to 6791 ng/g dw). This can mean that the sludge collected on the 16th of May is not in the same cycle as the one collected on the 22nd, proving that this last one was probably a fresh new sludge compared with the last cycle. Thus, knowing that a cycle corresponds to 15 - 16 days, the sample collected on 29th will be in the same cycle as the one taken on 22nd. As expected, the siloxanes concentration decreased (from 6791 to 2991 ng/g dw). On the 5th of June, concentrations increased once again (2991 to 3805 ng/g dw), having the latter sample similar concentrations to those found in the sample taken on 12th of June (4211 ng/g dw). Thus, it can be assumed that the last collected sludges were in the same cycle. Once more, D5 was the predominant cVMSs (2265 to 4983 ng/g dw), followed by D6 (from 495.8 on 29th of May to 1244.1 ng/g/dw on 22nd of May).

5.3.4.2 Sludge dewatering

To check differences between the upcoming digested sludge with the dewatered sludge, samples were collected in the centrifuge (responsible for the mechanical dewatering), immediately after the discharge from the digester. The main results are present in Appendix 7 Sample Analysis, Table A21 and Figure 17.

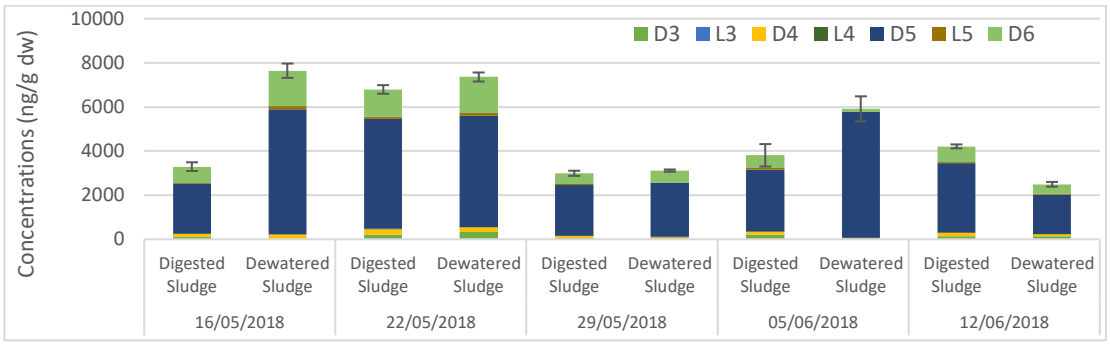


Figure 17 Comparison of the VMSs concentrations in the sludge before and after dewatering treatment. Error bars correspond to standard deviations (n=2).

Overall concentrations increased after the dewatering process (for example in the 16th of May increased from 3292 to 7842 ng/g dw). This can suggest that when the sludge reaches the dewatering treatment, some of the biogas trapped inside the sludge may sorb again (Crone et al., 2016; Al-mashhadani et al., 2016). Consequently, the VMSs present in that biogas may be bind to EPS, enriching its concentration in the final matrix. The sample collected on the 12th of June did not follow the general behaviour, possibly related with the weather conditions, since the temperature was higher in that day, favouring the volatilization of the studied compounds. Also, it was noticeable that the biological activity has increased due to this higher temperature, leading to observed fungi contamination, which might have interfered with the chemicals degradation.

Once more, D5 was detected in higher concentrations (from 17823 to 5703 ng/g dw).

5.3.4 Overview of the whole treatment process

Analysing the composite samples from the primary settler (1175 - 2602 ng/g dw) and comparing them with the total VMSs concentrations in the final sludge (3112 - 7642 ng/g dw), it is obvious that their concentrations increase along the wastewater treatment. This means that VMSs, with the treatment process, are accumulating and being absorbed into the solids. Among VMSs, cVMSs were the predominant compounds, especially D5 and D6, being always present in higher concentrations than linear VMSs. This might suggest that influents coming from a domestic source are the dominant in this WWTP, due to its extent use in daily commodities and health care products, against to lVMSs, which are often use in industrial products (Bletsou et al., 2013).

Now, comparing the results obtained for the primary sludge with those found in other studies, it can be seen that higher levels were found in the present study (nd (D3 and L3) to 1250 ng/g dw (D5)) than in Zhang (2014), whose levels ranged from 0.0003 (D4) to 0.007 (D5) ng/g dw. This can mean that in different regions, the population habits differ and, consequently, the personal care products used, which can influence VMSs concentration that reach the influent of the WWTPs.

Different concentrations of VMSs in secondary sludge were also found in other studies. For example, Dewil et al. (2007) reported concentrations between 0.575 and 253.3 (D5) ng/g dw, Wang et

al. (2015) concentrations from 1.27 (L4) to 4170 (D5) ng/g dw and Li et al. (2016) reported concentrations from nd (L5-L14) to 15000 (D5) ng/g dw. In this study, VMSs concentrations varied from nd (D3 and L3) to 6384 (D5) ng/g dw being, therefore, in the same range of concentrations as those reported by Wang et al. (2015). Dewil et al. (2007) showed lower concentrations, maybe due to the type of influent received in this WWTP. Li et al. (2016) described higher concentration, namely for D5, which was already explained by the author - lack of restrictions of these compounds on PCPs in China, compared with the other countries.

The concentrations of VMSs in digested sludge were also reported in the literature. Zhang (2014) showed VMSs concentration from 0.0004 (D4) to 0.0234 ng/g dw (D5) and Oshita et al. (2014) from nd for D4 to 0.84 ng/g dw for D5. Comparing this study with the abovementioned, the digested sludge investigated presented higher levels, varying from nd (L3) to 4983 ng/g dw (D5). High concentrations of the cVMSs should be explained due to the type of influent, which is predominantly domestic. D5's concentration remains almost invariable during the WWTP, being only part of it volatilized in the anaerobic treatment, which demonstrates its biopersistent and bioaccumulating character.

The concentrations in the final digested dewatered sludge in other studies ranged in Zhang (2014) from 0.001 to 0.0314 ng/g dw (D5), in Li et al. (2016) from nd to 10900 ng/g dw for L5 and D5 respectively, in Bletsou et al. (2013) from 7 (D3) to 17500 (D5) ng/g dw, in Liu et al. (2014) from <LOQ (all VMSs) to 36000 (cVMSs), while in this study varied from nd (D3 and L3) to 5703 (D5) ng/g dw. This study shows that, in general, lower concentrations are achieved in this WWTP. Although, concentrations found in this study are lower than the ones presented before, they are still a reason to raise concern since they may represent an environmental problem as well as a public health issue.

Two other sludge samples coming from Chile (dried in an oven during 48 h) and Aveiro (80% water content; removed with the freeze-drier), from the dewatering process, were also extracted in this study, in the same way as those from Matosinhos. Concentrations are presented in Table A22, Appendix 7 Samples Analysis. Higher concentrations were achieved in Aveiro (nd - 3139 ng/g dw) compared with Chile (nd - 795 ng/g dw). The results were in accordance with previous studies, being cVMSs the most predominant, where D5 (ranged from 795 and 3139 ng/g dw, in Chile and Aveiro respectively) and D6 (260 and 1559 ng/g dw, in Chile and Aveiro respectively), were the major contributors. Regarding lVMSs, concentrations were higher for L5 (8 and 72 ng/g dw for Chile and Aveiro respectively) and nd for L3 in both samples. Given the fact that the sample treatment was different (the sludge from Aveiro was freeze-dried, while those from Chile had already been subjected to a drying process by heat), the VMSs concentration may have been affected and therefore, this may justify the lowest concentration values in the sample from Chile. Still, the VMSs concentration are lower than in the studied WWTP. Due to the lack of knowledge about the sludge matrix treatment in other studies (Tavazzi et al., 2012; Companioni-Damas et al., 2012; Kaj et al., 2005; Zhang et al., 2010), concentrations were not compared with the obtained results from this study.

6 Conclusions

Sludge is a common matrix for analysis of organic pollutants as it receives a fair amount of chemicals used in households and consumer products, as well as in industrial processes. Numerous substances have been detected in sludge in concentrations high enough to raise concern, since it may be used as fertiliser in agriculture, due to several advantages of nutrient and organic matter recycling. In addition, the responsible for WWTPs are becoming more aware of the problems related to VMSs persistence, due to the environmental issues as well as the equipment maintenance costs. Therefore, it is important to understand at which levels these pollutants can be found in sludge samples. For that, a protocol based on solid-liquid extraction followed by gas chromatography-mass spectrometry was adapted from previously described studies (Bletsou et al., 2013; Liu et al., 2014) to determine VMSs (L3-L5, D3-D6) in sludge samples. The proposed method was validated and the linearity, limits of detection and quantification were determined, as well as the precision (intra- and interday; relative standard deviations < 30%) and the accuracy of the method (69±6% to 124±10%).

To better understand the impact of siloxanes in a WWTP, assays were performed in a WWTP located in Matosinhos, Portugal. To our best knowledge, this is the first study analysing VMSs in a Portuguese WWTP and also the first one reporting their concentrations along the sludge line.

Analysing the whole treatment, higher concentrations were reached in the biological reactor (mean concentrations varied between 4873 to 8059 ng/g dw). This may be related to the EPS formation, which enhance VMSs concentrations in the matrix. cVMSs were dominant in all the samples, being around 98% of the total VMSs concentration. D5 was the major contributor in all the units (its presence varied from approximately 57% in the gravitational thickener to 82% in the mechanical thickener). Higher concentrations were found in other studies, where D5 was also the major compound. This may indicate that D5 is stable in WWTP and its sorption to sludge hampers volatilization.

The use of these siloxanes is continuous and, from a perspective of circular economy, the reuse of sludge has been encouraged (e.g. as agricultural fertiliser). This can lead to increase their levels in the environment, reaching potentially harmful concentrations. Therefore, siloxanes may be a barrier to the use of municipal sewage sludge in a sustainable way.

Furthermore, the fate of VMSs should be investigated in more detail, in order to evaluate if the siloxanes end-up in the biogas (being able of damaging biogas engines), to infer the quantity that volatilize into the atmosphere, the quantity of siloxanes that does not sorb in the sludge, remaining in the wastewater and the amount of siloxanes that ends-up to be biochemically decomposed.

7 Limitations and Future Work

Due to technical issues in the GC-MS and this equipment being shared with other members of the technical team, to the long steps on the treatment of the samples and extraction until further analysis on the instrumental equipment, and also since the WWTP was in a start-up phase, the schedule and analysis of the experimental work was changed and planned with belated samples, being impossible to include the last collected samples. Besides, some samples were not taken due to technical issues.

The analysis of siloxanes is a difficult task, since these compounds are volatile, which sometimes results in its lost during laboratory experiments and also, they are present in the surrounding environment, being difficult to achieve a total chemical aseptic condition, with the available resources. Besides, the sludge matrix is also a barrier in the analysis of these compounds, since it is not easy to homogenise (due to existing hair, sticks, rocks, etc) and the fact that weather conditions are changeable (rain and high temperatures), sometimes they end-up to be more diluted or volatilized, being hard to pursue its course during the WWTP sludge line. Also, it was noticeable that inside the containers, during transportation, some gas was produced, which might result in siloxanes volatilization and lost. Some methods could be explored to achieve a better and quick homogenization of the sample, namely thermal drying at low pressure (ensuring that less temperatures would be required to reduce the sludge until dust) and also the freezing of the sludge through liquid nitrogen, which would be a quicker way of obtaining small particles. Also, the usage of a clean air room or chamber would be an improvement, in order to reduce potential chemical contaminations of samples.

Other internal standards could be tested, namely deuterated siloxanes or ^{13}C-labelled siloxanes since it was observed some inconsistency and lack of reproducibility in M4Q for same samples. The implementation of a clean-up step would be also an improvement of the method, as well as changing conditions on GC-MS (oven temperatures, injection volume, etc) to see if it would affect or not the overall results, achieving more reliable conclusions.

To finish, it would be interesting to assess variations of VMSs: along the seasons; in different WWTP, where different treatments took place and receiving different types of influents; in wastewater lifting stations, to infer which cities would influence more the total results; in the biogas and in the water matrix, in order to obtain more conclusions on the fate of VMSs through a mass balance; in the soil, after application of the sludge, to obtain VMSs concentration and in the food, to see if persistence of the compounds would be impactful in the food chain and, therefore, in the public health. More linear VMSs could be also explored, regarding previous studies, where its presence was assessed.

It would be also interesting to study some technologies in order to prevent siloxanes presence in the biogas and in the sludge (namely using microorganisms for their degradation).

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Appendix 1 Ecotoxicity

To lead to some conclusions of the toxicological effect of D4 and D5, already discussed in the Section 2 (2.2.4 Environmental Concern and Ecotoxicity), some studies were conducted in rats and humans, by applying different concentrations in the stated species:

- Following topical application between 1 and 24 hours, a percutaneous absorption of neat D4 in humans has been shown at levels of 0.57–1.09% ((EPA DCN 86980000153, 1998; EPA DCN 86010000003, 2000) reported in (Lassen et al., 2005)) and, following 24 hours exposure, in in vitro studies, with percutaneous absorption to 14C-D5, the absorption was found to be 0.8–1.08% ((EPA DCN 86970000009, 1996c; EPA DCN 86960000593, 1996b) mentioned in (Lassen et al., 2005)).
- An investigation of subacute oral toxicity in rats, administered between 25 and 1600 mg/kg per gavage over two weeks, with five applications per week, resulted in an increase of both relative liver weight (in female animals in 100 mg/kg and male animals in 400 mg/kg) and absolute liver weight (female rats at 400 mg/kg). At the highest concentration the body weight decreased in male and female (Lassen et al., 2005).
- Rats were also exposed to D4 at 70 and 700 ppm by inhalation for 28 days, 5 days per week and 6 hours per day. This resulted in a rapid, but reversible, increase in liver size and in an induction of several metabolising enzymes. As for D5 inhalation (during 28 days, 7 days per week and 6 hours per day) at concentrations between 10 and 160 ppm showed no adverse effects on body weight, food consumption or urinalysis. Only at 160 ppm were observed small changes in 48 haematological serum chemistry and organ weight and a transient increase in liver to body weight and thymus to body weight ((McKim et al., 1998; EPA DCN 86970000723, 1996a) mentioned in (Lassen et al., 2005)). Sub-chronic toxicity studies in rats exposed over three months at doses up to 224 ppm show that the lung is the primary target organ, following D5 inhalation ((Burns-Naas et al., 1998) mentioned in (Lassen et al., 2005)).

In aquatic ecosystems, studies for the same cVMSs (D4 and D5) were also carried (Wang et al., 2013):

- For the midge, using 14-d aqueous exposures at five concentrations ranging from 0.49 to 1 µg/L. No adverse effects were observed. But, the investigation of the acute and chronic toxicity of D4 to some representative freshwater and marine fish and invertebrates and observed rainbow trout noted that these are most sensitive to this component. For small-sized (⩽1 g) rainbow trout, the lowest concentration causing 50% mortality (LC50) in a 14-d

acute test was 10 µg/L, with a non-observed-effect-concentration (NOEC) of 4.4 µg/L and a lowest-observed-adverse-effect-concentration (LOAEC) of 6.9 µg/L. The chronic NOEC was 4.4 µg/L in a 93-d exposure of rainbow trout early life stages, the same as determined in the 14-d test above. D4 caused significant mortality at 15 µg/L during a 21-d chronic toxicity study with daphnids.

- As for D5, juvenile rainbow trout were exposed at 2.1, 3.1, 5.0, 8.6, and 16 µg/L for 14-d under a flow-through system; no significant mortality was observed, and the acute and chronic toxicity studies indicate that D5 does not exhibit adverse effects on fish and water flea exposed at concentrations up to its water solubility limit (17 µg/L). It was observed that D5 toxicity to *H. Azteca* increased with the decreasing sediment organic matter content due to increased bioavailability.

Regarding the study about D5 in the soil (Velicogna et al., 2012):

D5 was spiked into a surrogate biosolid and then mixed with a sandy loam soil to create test concentrations ranging from 0 to 4074 mg/kg. Toxicity tests were made to evaluate lethal and sub-lethal effects in plant (*Hordeum vulgare* (barley) and *Trifolium pratense* (red clover)) and soil invertebrates (*Eisenia andrei* (earthworm) and *Folsomia candida* (springtail)). Plant testing evaluated the effects on seedling emergence, shoot and root length, and shoot and root dry mass. Invertebrate test endpoints included adult lethality, juvenile production, and individual juvenile dry mass (earthworms only). Also, to track back concentrations and assess the loss of the compound over the duration of a test, soil samples were collected from time to time. D5 losses of up to 50% were observed, specially at high concentrations. Higher concentrations of D5 were selected to increase the likelihood of observing an effect on organisms in order to determine its ecotoxicity in them. Despite this, in many of the test endpoints measured, particularly for *E. andrei* and *T. pratense*, no significant adverse effects were observed, contrarily to *F. candida* (adult survival and juvenile production) and *H. vulgare* (shoot length and dry mass and root dry mass), with toxicity estimates (IC50) ranging from 209 to 2051 mg/kg.

Appendix 2 Biogas Purification Techniques

Table A1 Compilation of techniques employed to remove siloxanes from biogas produced in WWTP facilities.

	Techniques	Descriptions (Advantages/Disadvantages)	Reference
Adsorption	Activated Carbon Cocoa shell Bituminous coal Silica gel Molecular sieves	All are good for the removal of D5 Activated carbon is more efficient with a pre-drying step Cocoa shell and bituminous coal are less expensive and more often used in industry Silica gel is the most cost-effective, good regeneration (especially for L2)	(Ruiling et al., 2017)
Absorption	*Chemical:* Strong acids and bases Phosphoric acid Nitric acid Sulphuric acid *Physical:* Water Organic solvent (Selexol™) Mineral oil	 Acidic liquids are the most feasible ones, not forming other components through reactions The elimination rates are better for sulphuric acid (approximately 95%) Acids are corrosives and non-safe methods Most siloxanes are hydrophobic. Water can be pre-processing step for soluble contaminants Rates of 99% can be achieved for organic solvents Oil removes 60% These techniques include spray and packed columns The siloxanes are easily stripped at a high gas flow rate	(Ruiling et al., 2017; Soreanu et al., 2011)
Cryogenic condensation	Lower Temperatures 5º C -25º C -70º C	The more we lower the temperature, better removal is obtained and more expensive is the method Is not easy to remove L2, D3 and L3, due to the condensation point -25º C is the optimal used in industry, in combination with adsorption	(Ruiling et al., 2017)
Catalytic Process	Alumina V_2O_5-TiO_2	Deactivates overtime; requires replacement Good activity for oxidation/hydrolysis Optimal temperature of the process is 250-400 ◦C	(Ruiling et al., 2017)
Biological removal	Biofiltration systems Pseudomonas Rhodanobacter, Zooglea, Mesorhizobium, Xanthomonadacea	D4 and D5 have 44 and 62% degradation ratio Mass transfer limitations Poor degradation	(Soreanu et al., 2011; Ruiling et al., 2017)
Membrane separation	Polymeric Membrane material PDMS	Good removal Still under study Requires compressors and vacuum pumps	(Soreanu et al., 2011)

Appendix 3 GC-MS

A number of options are available for GC injection systems, which allow the introduction of the sample into the equipment (Courant et al., 2007) . The choice of optimum sample introduction strategy depends on the concentration range of target analytes, their physicochemical properties and on the occurrence of matrix co-extracts present in the sample (Hinshaw et al., 2009). The most widely used option is the split/splitless injector (Figure A1). With splitless injection, the injector temperature should be high enough to volatilize all the analytes. After injection, the sample is directed introduced into the column (Douglas, 2011). The compounds comprising the mixture of interest are separated by their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase) and specially according to their boiling points. As the components become separated, they elute from the column at different times, which is generally referred to as their retention times (Bul, 2008).

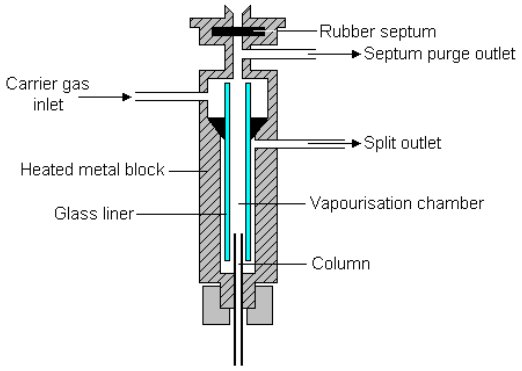

Figure A1 The split/splitless injector of the gas chromatograph (Sheffield Hallam University, 2017).

There are two types of columns used in GC: packed and open tubular, also known as capillary column. Capillary columns are frequently used due to their separation efficiency. The boiling point of the solvent and analytes defines the optimal column temperature and the choice of the most suitable stationary phase that must reveal some affinity for the analytes (Skoog et al., 2007). A carrier gas (usually helium) is used to transport the sample species through the column (Bul, 2008).

The latter part of the column passes through a heated transfer line and ends at the entrance to ion source where compounds eluting from the column are converted to ions. Two potential methods exist for ion production as mentioned before. The most frequently used method is electron ionisation (EI) and the occasionally used alternative is chemical ionisation (CI). For EI a beam of electrons ionises the sample molecules resulting in the loss of one electron (Figure A2). When the resulting peak from this ion is seen in a mass spectrum, it gives the molecular weight of the compound. Due to the large amount of energy imparted to the molecular ion it usually fragments producing further smaller ions with characteristic relative abundances that provide a 'fingerprint' for that molecular structure. This

information may be then used to identify compounds of interest and help elucidate the structure of unknown components of mixtures. Summarizing, ions are separated based on their different mass-to-charge (m/z) ratios (Bul, 2008; EAG Laboratories, c2017).

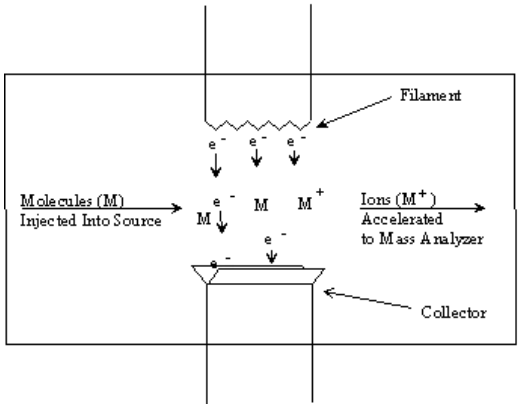

Figure A2 Electron Ionization Source (Bramer, 1997).

The next component is a mass analyser (filter), which separates the positively charged ions according to various mass related properties depending upon the analyser used. Several types of analyser exist. The most common are quadrupoles and ion traps. After the ions are separated they enter a detector the output from which is amplified to boost the signal. The detector sends information to a computer that records all the data produced, converts the electrical impulses into visual displays and hard copy displays. In addition, the computer also controls the operation of the mass spectrometer (Bouchonnet, 2013; Bul, 2008).

Appendix 4 Sampling strategy and water content

The figure below shows the sampling points where the different types of sludge were collected for further analysis, as well as the respective sludge retention times (SRT).

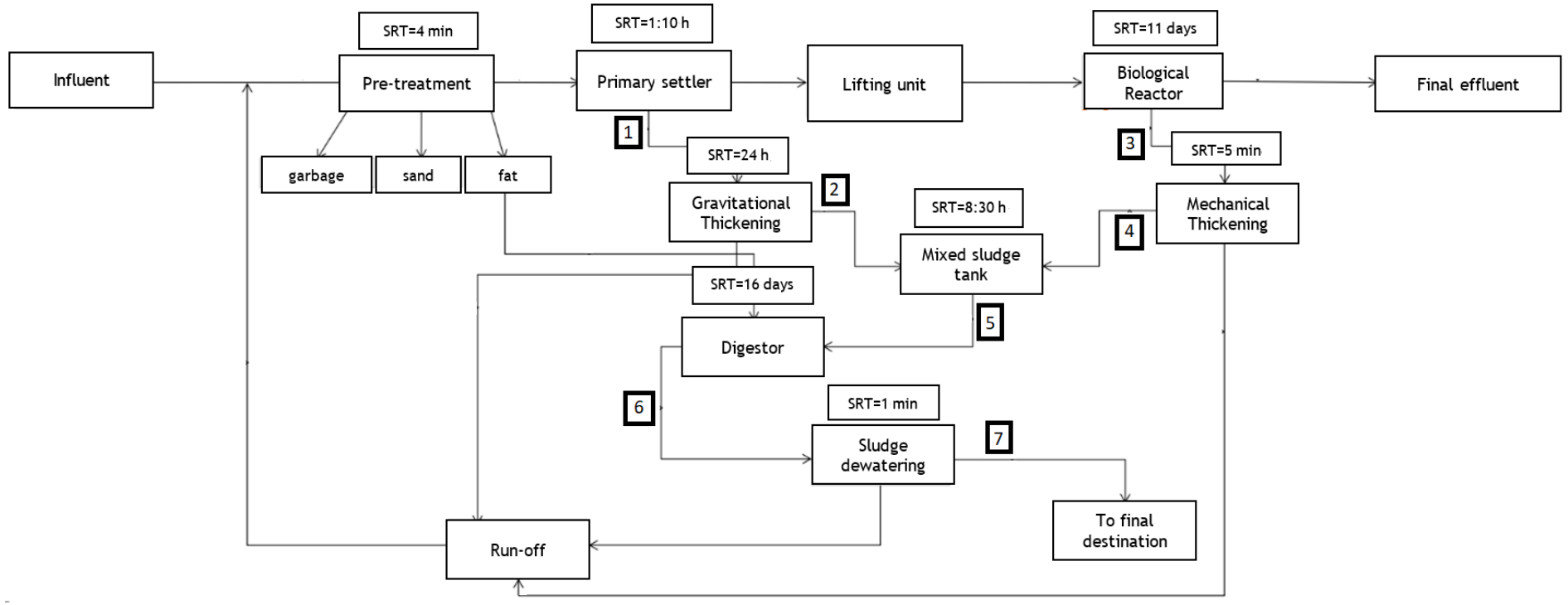

Figure A3 Scheme of the Matosinhos WWTP: the numbers represent the location of the collected sludge samples (adapted from (Sisaqua, 2016).

A schedule, to assess VMSs concentration in the WWTP, was defined according to the SRT in each treatment step (Table A2).

Table A2 Plan of the sludge collection, according with the SRT and explanation of the choice of dates.

Unit	SRT	Dates	Explanation
Primary Settler	1.17 h	15th to 21st of May	Grab samples collected during 7 consecutive days, every 4 hours, to record variations on the VMSs concentrations among a day. Composite samples were also performed in laboratory in order to compare the concentrations of VMSs with the results from the next unit.
Gravitational Thickener	24 h	16th to 22nd of May + 12th of June	Grab samples collected at 3 am to compare the influence of the thickening treatment (comparison with composite samples). The last sample was collected to compare with the wastewater that was sent to the Biological Reactor and that will leave the unit 11 days later.
Biological Reactor	11 days	16th of May + 21st of May + 2nd of June + 13th of June + 22nd of June	The first two samples were taken with 5 days interval, to gauge the variability of the VMSs concentration in the sludge from the same cycle (half of SRT - 5 days) and then, every 11 days (equivalent to the SRT), to make sure that the collected samples were not the same. Last sample will have the same influent as the 12th of June of the last unit, as explained already. All the samples were taken at 10:00 am.
Mechanical Thickener	5 min	Same dates as in the unit before	To observe the influence of the thickening process in VMSs concentrations. All the samples were collected at 10:05 am.
Mixed Tank	8.5 h	16th to 18th of May + 12th of June	First sample makes possible a mass balance in that reactor. Other samples used to study the gravitational thickener process and possible variations of the VMSs concentrations. Samples were collected at 12:30 pm.
Digester	15 days	16th, 22nd and 29th of May + 5th and 12th of June	To study possible variations inside the digester, assuring that sludge from two different cycles were taken. The grab samples were collected at 10:30 am.
Centrifuges	1 min	The same dates as in the unit before	To study possible variations in the dewatering process.

The water content was assessed by the following equation:

$$Water\ content\ (\%) = \frac{initial\ mass\ sludge - final\ mass\ sludge}{initial\ mass\ sludge} \times 100 \quad \text{(Equation 1)}$$

Appendix 5 Significance tests

In statistical significance testing, a one-tailed test and a two-tailed test are alternative ways to test whether results are that different from each other and, therefore, evaluate if the method can or cannot be applied (H_0=0).

Resorting to an Excel tool from the Analysis ToolPak, and assuming that we have two different distributions of samples with different variances, two-tailed test were made, to see if the recovery results depend on the sample treatment – sieved or milled + sieved (Pearson correlation, p>0.05). For each siloxane, and according with the obtained recoveries from the both sample treatment (Table A3), the *t*-tests are shown from Table A4-A10.

Table A3 Recoveries of sieved (n=3) and milled + sieved samples (n=3).

SAMPLE TREATMENT	SIEVED	MILLED + SIEVED
COMPOUND	**Recoveries (%)**	
D3	50	53
	54	51
	52	50
L3	37	35
	36	36
	32	33
D4	91	85
	93	86
	87	84
L4	101	97
	103	97
	98	94
D5	99	91
	86	84
	77	86
L5	106	103
	104	102
	103	100
D6	100	98
	94	96
	92	97

Table A4 *t*-test of the recoveries for D3, for the sieved and milled + sieved samples.

D3	*Sieved*	*Milled*
Mean	0.521124	0.514993
Variance	0.000374	0.000261
Observations	3	3
Grouped variances	0.000318	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.421374	
P(T<=t) two-tail	0.695139	
t Critical two-tail	2.776445	

Table A5 *t*-test of the recoveries for L3, for the sieved and milled + sieved samples.

L3	*Sieved*	*Milled*
Mean	0.350937	0.347377
Variance	0.000529	0.000176
Observations	3	3
Grouped variances	0.000352	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.232288	
P(T<=t) two-tail	0.827715	
t Critical two-tail	2.776445	

Table A6 *t*-test of the recoveries for D4, for the sieved and milled + sieved samples.

D4	*Sieved*	*Milled*
Mean	0.901851	0.849996
Variance	0.000957	0.000164
Observations	3	3
Grouped variances	0.00056	
Hypothesized Mean Difference	0	
df	4	
t Stat	2.682725	
P(T<=t) two-tail	0.055072	
t Critical two-tail	2.776445	

Table A7 *t*-test of the recoveries for L4, for the sieved and milled + sieved samples.

L4	*Sieved*	*Milled*
Mean	1.007772	0.959846
Variance	0.00064	0.000216
Observations	3	3
Grouped variances	0.000428	
Hypothesized Mean Difference	0	
df	4	
t Stat	2.836114	
P(T<=t) two-tail	0.047052×	
t Critical two-tail	2.776445	

Table A8 *t*-test of the recoveries for D5, for the sieved and milled + sieved samples.

D5	*Sieved*	*Milled*
Mean	0.875192	0.86937
Variance	0.012639	0.001193
Observations	3	3
Grouped variances	0.006916	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.085738	
P(T<=t) two-tail	0.935795	
t Critical two-tail	2.776445	

Table A9 *t*-test of the recoveries for L5, for the sieved and milled + sieved samples.

L5	*Sieved*	*Milled*
Mean	1.044756	1.01875
Variance	0.000272	0.000335
Observations	3	3
Grouped variances	0.000304	
Hypothesized Mean Difference	0	
df	4	
t Stat	1.827706	
P(T<=t) two-tail	0.141604	
t Critical two-tail	2.776445	

Table A10 *t*-tests of the recoveries for D6, for the sieved and milled + sieved samples.

D6	*Sieved*	*Milled*
Mean	0.951721	0.974018
Variance	0.001897	0.000123
Observations	3	3
Grouped variances	0.00101	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.859377	
P(T<=t) two-tail	0.438586	
t Critical two-tail	2.776445	

Appendix 6 Method Validation

The calibration curves (Figure A4 to A10) were plotted for each compound based on the Response Factors (RF), shown in Table A10, at different concentration levels. The respective validation parameters are also shown in Table A11, where the calculated parameters, a and s_a, (equation 2 and 3) were based on Eisenhauer (2003).

Table A11 Concentrations and response factors (RF) for each studied VMS.

			RF						
C (µg/L)	**$m_{injected}$ (ng)**	**$(m/m_{IS})_{injected}$**	**D3**	**L3**	**D4**	**L4**	**D5**	**L5**	**D6**
5	0.005	0.02	0.08	0.02	0.04	0.02	0.02	0.03	0.02
10	0.010	0.04	0.08	0.03	0.06	0.04	0.04	0.05	0.04
50	0.050	0.20	0.26	0.17	0.26	0.20	0.20	0.27	0.17
100	0.100	0.40	0.42	0.33	0.53	0.40	0.41	0.52	0.34
250	0.250	1.00	0.93	0.80	1.24	0.96	0.98	1.29	0.77
500	0.500	2.00	1.65	1.43	2.21	1.70	1.76	2.30	1.42
750	0.750	3.00	2.62	2.31	3.62	2.74	2.90	3.81	2.30
1000	1.000	4.00	3.21	2.80	4.42	3.34	3.52	4.65	2.86
1250	1.250	5.00	3.99	3.52	5.44	4.14	4.51	5.85	3.65
1500	1.500	6.00	5.12	4.44	7.02	5.38	5.71	7.38	4.75
IS (250)	**0.250**	**1.00**							

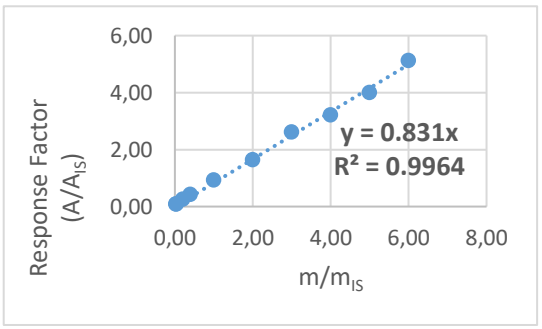

Figure A4 Calibration curve for D3 using GC-MS.

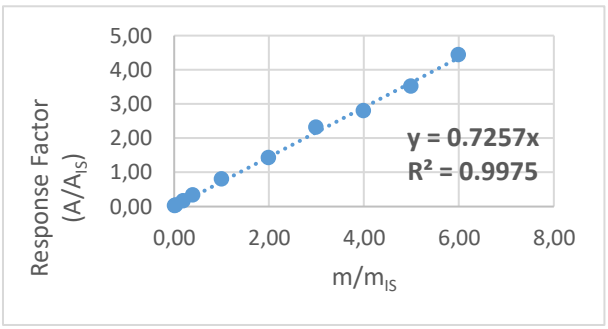

Figure A5 Calibration curve for L3 using GC-MS.

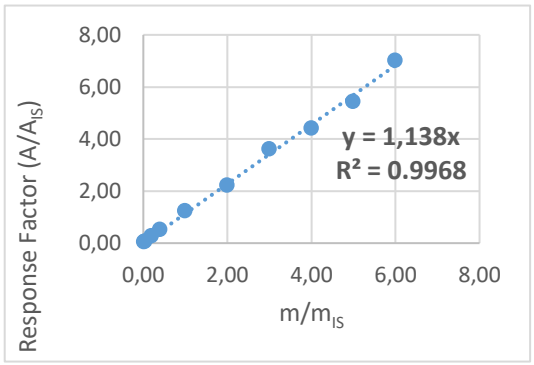

Figure A6 Calibration curve for D4 using GC-MS.

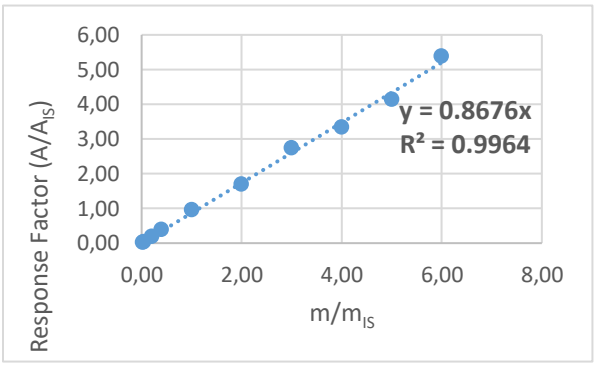

Figure A7 Calibration curve for L4 using GC-MS.

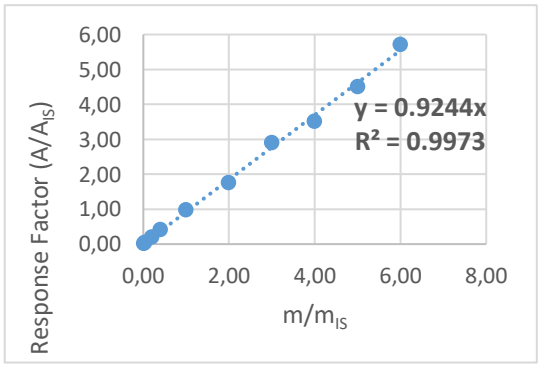

Figure A8 Calibration curve for D5 using GC-MS.

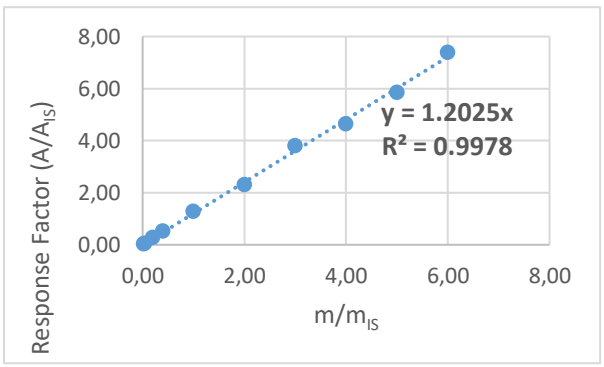

Figure A9 Calibration curve for L5 using GC-MS.

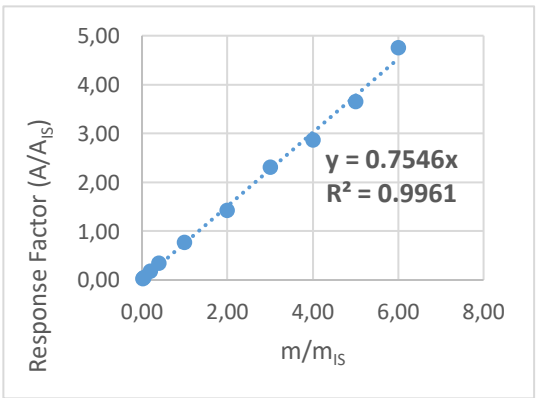

Figure A10 Calibration curve for D6 using GC-MS.

Table A12 Validation criteria: linearity response, (*r*) and relative standard deviations of the slope (s_a/a).

Compound	*r*>0.995	s_a	$s_a/a \times 100 < 5\%$
D3	0.998	0.011	1.4
L3	0.999	0.008	1.1
D4	0.998	0.015	1.3
L4	0.998	0.012	1.4
D5	0.999	0.011	1.2
L5	0.999	0.013	1.1
D6	0.998	0.011	1.5

$$a = \frac{\sum x_i\, y_i}{\sum x_i^2} \ (Equation\ 2)$$

Where a is the slope of the obtained calibration curves

$$s_a = s_{0,0} \times \frac{\sqrt{\sum x_i^2}}{\sum x_i^2}, where\ s_{0,0} = \sqrt{\frac{\sum (x_i - a x_i)^2}{n-1}} \ (Equation\ 3)$$

Where s_a is the error associated to the slope and $s_{0,0}$ is the square root of the quantity found by dividing the sum of the squares of the deviations from the best fit line, by the number of data points you beyond the minimum required only one additional point is needed to draw a straight line through the origin) to fit the specified curve

Appendix 7 Samples Analysis

Primary Treatment

In order to study the behaviour of siloxanes during a week and also to see if there were variations along the day, sludge samples were collected from the primary settler, every 4 hours, during a week. Results are expressed in the Table A13.

Table A13 Siloxanes concentration in the sludge (ng/g dw) during a week on the primary settler, every 4 hours.

	Tuesday 15/05/2018				Wednesday 16/05/2018						Thursday 17/05/2018					
Compound	**9 h**	**13 h**	**17 h**	**21 h**	**1 h**	**5 h**	**9 h**	**13 h**	**17 h**	**21 h**	**1 h**	**5 h**	**9 h**	**13 h**	**17 h**	**21 h**
D3	13.5	67.5	nd	20.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
D4	84.2	97.8	54.0	51.9	37.7	28.5	36.0	43.3	17.0	15.3	21.7	33.7	32.7	316.6	30.3	28.6
L4	3.6	3.2	3.0	2.2	3.6	4.0	4.1	3.2	3.0	1.7	2.7	3.8	3.6	6.3	2.7	2.2
D5	864.3	846.5	709.8	588.9	937.1	981.7	1033.0	647.8	574.5	331.0	635.8	1286.2	911.0	1607.9	559.4	480.2
L5	17.1	17.9	16.9	13.8	18.8	17.8	19.9	14.9	14.3	8.5	16.8	43.2	21.8	31.9	12.4	10.2
D6	401.5	219.6	200.8	398.7	260.8	231.3	298.4	186.8	166.2	104.7	225.4	918.3	314.5	451.7	144.6	126.8
Total	**1384.3**	**1252.5**	**984.4**	**1075.6**	**1258.1**	**1263.3**	**1391.5**	**896.0**	**774.9**	**461.3**	**902.3**	**2285.3**	**1283.7**	**2414.5**	**749.3**	**648.0**

Table A13 Siloxanes concentration in the sludge (ng/g dw) during a week on the primary settler, every 4 hours (cont.).

	Friday 18/05/2018						Saturday 19/05/2018						Sunday 20/05/2018					
Compound	**1 h**	**5 h**	**9 h**	**13 h**	**17 h**	**21 h**	**1 h**	**5 h**	**9 h**	**13 h**	**17 h**	**21 h**	**1 h**	**5 h**	**9 h**	**13 h**	**17 h**	**21 h**
D3	nd	nd	nd	11.4	17.5	nd	102.8	112.2			355.6	84.8			nd	nd	212.3	222.1
L3	nd	nd	nd	nd	nd	nd	nd	nd			nd	nd			nd	nd	nd	nd
D4	53.0	142.1	64.1	53.8	44.2	20.6	95.7	74.8			125.5	79.5			58.4	45.8	137.9	82.5
L4	3.1	14.8	7.1	3.7	3.5	1.6	4.8	3.8			3.5	3.7			3.3	5.9	4.8	3.2
D5	815.5	4095.5	2003.7	900.4	818.2	415.5	1123.7	847.8			868.9	928.8			888.4	738.0	1077.0	854.6
L5	16.4	152.8	49.5	20.2	18.8	8.4	24.3	17.1			17.4	16.3			17.5	17.1	22.1	16.6
D6	190.1	1630.6	643.0	265.0	269.2	118.4	332.4	260.7			234.2	243.4			198.3	175.8	254.0	241.1
Total	**1078.2**	**6035.8**	**2767.4**	**1254.5**	**1171.4**	**564.5**	**1683.7**	**1316.4**	**-**	**-**	**1605.1**	**1356.4**	**-**	**-**	**1165.9**	**982.6**	**1708.2**	**1420.1**

Table A13 Siloxanes concentration in the sludge (ng/g dw) during a week on the primary settler, every 4 hours (cont.).

	Monday 21/05/2018						Tuesday 22/05/2018	
Compound	**1 h**	**5 h**	**9 h**	**13 h**	**17 h**	**21 h**	**1h**	**5h**
D3	208.7	697.7	648.6	209.0	324.6		331.7	427.3
L3	nd	nd	nd	nd	nd		nd	nd
D4	87.8	190.0	236.8	146.3	231.1		131.7	206.5
L4	3.8	4.3	5.8	11.8	10.4		3.9	4.1
D5	946.9	1010.6	1638.7	2788.7	2031.4		880.3	1046.4
L5	18.7	19.4	32.8	66.2	42.3		16.8	19.4
D6	224.3	239.8	418.1	782.8	543.9		243.6	268.8
Total	**1490.2**	**2161.8**	**2980.8**	**4004.7**	**3183.7**	**-**	**1608.0**	**1972.5**

Besides, composite sludge samples were prepared in laboratory and an estimation of the mean concentrations for compound per day was calculated. Results of the expected concentrations and the obtained from the laboratory are presented in Table A14 and A15, respectively.

Table A14 Mean estimated concentrations of siloxanes (ng/g dw) based on the sludge taken every 4 hours, from the primary settler.

Compound	Tuesday 15/05/2018	Wednesday 16/05/2018	Thursday 17/05/2018	Friday 18/05/2018	Saturday 19/05/2018	Sunday 20/05/2018	Monday 21/05/2018
D3	20.2	0.0	0.0	21.9	184.2	128.6	419.6
L3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D4	65.1	27.0	82.5	70.1	93.2	82.5	183.7
L4	3.1	3.1	3.6	5.9	3.6	4.2	7.2
D5	789.3	700.7	943.4	1559.5	881.8	901.0	1667.7
L5	16.9	15.4	22.7	45.7	17.0	18.4	35.3
D6	296.3	202.1	357.7	543.1	246.1	218.7	443.4
Total	**1191.0**	**948.2**	**1409.8**	**2246.2**	**1426.0**	**1353.4**	**2756.9**

Table A15 Actual result concentrations of VMSs (ng/g dw) from composite samples made in the lab.

Compound	Tuesday 15/05/2018	Wednesday 16/05/2018	Thursday 17/05/2018	Friday 18/05/2018	Saturday 19/05/2018	Sunday 20/05/2018	Monday 21/05/2018
D3	81.5	nd	nd	263.6		397.5	nd
L3	0.0	0.0	0.0	0.0		0.0	0.0
D4	72.4	69.7	75.2	233.1		236.4	156.0
L4	2.6	3.1	3.0	4.4		4.2	7.2
D5	803.3	820.9	906.3	1250.2		1050.1	1907.3
L5	20.8	19.5	23.4	31.2		26.1	45.2
D6	301.1	261.8	392.4	350.4		319.5	488.6
Total	1281.9	1175.1	1400.4	2132.9		2033.8	2604.2

Grab samples were also taken at 3 am from the gravitational thickener, during a week and concentrations of the target analytes were determined. Table A16 sums ups these results and each concentration day reflects the day before from the sludge taken on the primary settler.

Table A16 Concentrations of siloxanes in the gravitational thickened sludge (ng/g dw), during a week.

Compound	Wednesday 16/05/2018	Thursday 17/05/2018	Friday 18/05/2018	Saturday 19/05/2018	Sunday 20/05/2018	Monday 21/05/2018	Sunday 22/05/2018
D3	40.1	23.6	34.5		505.5	412.0	427.3
L3	nd	nd	nd		nd	nd	nd
D4	73.3	30.2	52.1		156.4	172.6	206.5
L4	3.3	3.0	2.7		3.3	5.7	4.1
D5	636.5	665.2	792.0		623.3	1569.5	1046.4
L5	15.8	15.4	18.5		13.2	39.5	19.4
D6	371.6	179.9	225.0		193.5	568.5	268.8
Total	**1140.6**	**917.4**	**1124.8**	**-**	**1495.2**	**2767.9**	**1972.5**

Secondary Treatment

From the secondary treatment, samples were collected from 16th of May at 10 am in the biological reactor (Table A17) and with a 5-minute delay in the mechanical thickener (Table A18) to observe if the treatment affects the VMSs concentration.

Table A17 Concentrations of the studied VMSs in the collected sludge from the biological reactor (ng/g dw).

Compound	16/05/2018	21/05/2018	02/06/2018	13/06/2018	22/06/2018
D3	89.8	nd	148.9	114.4	
L3	nd	nd	nd	nd	
D4	112.9	109.9	255.4	136.6	
L4	17.9	23.3	15.1	14.8	
D5	3976.2	6383.7	4297.3	3719.9	
L5	99.6	140.8	90.9	87.0	
D6	1166.4	1401.2	1026.9	800.6	
Total	**5462.8**	**8059.0**	**5834.5**	**4873.3**	

Table A18 Concentrations of the target analytes in the mechanical thickened sludge (ng/g dw).

Compound	16/05/2018	21/05/2018	02/06/2018	13/06/2018	22/06/2018
D3	43.0		nd	121.5	
L3	nd		nd	nd	
D4	153.0		111.6	144.3	
L4	20.4		22.1	15.4	
D5	4548.6		5702.3	3696.3	
L5	116.2		121.8	80.2	
D6	1360.4		132.7	745.3	
Total	**6241.6**	**-**	**6090.5**	**4803.0**	

Sludge Mixing

Samples were collected in the mixed sludge tank during three consecutive days - as the name suggest, the tank combines the primary and secondary sludge. Final concentrations are expressed in the Table A19.

Table A19 Concentrations of VMSs in the mixed sludge tank (ng/g dw).

Compound	Wednesday	Thursday	Friday	Tuesday
	16/05/2018	17/05/2018	18/05/2018	12/06/2018
D3	17.5	27.3	82.3	179.5
L3	nd	nd	nd	nd
D4	73.0	109.6	97.8	100.4
L4	4.8	21.6	12.2	7.1
D5	1147.2	5519.6	2947.7	1812.5
L5	26.4	134.9	67.1	42.2
D6	295.6	1328.9	732.8	420.4
Total	**1564.5**	**7141.9**	**3939.9**	**2562.1**

Anaerobic Digestion

In order to establish the concentration profile of siloxanes during the anaerobic treatment, samples were collected from the digester and the centrifuge. The sample on the 16th of May can either be in the cycle of the 22th of May or the 22th can be in the same cycle of the 29th of May. Results are shown below in Table A20 and A21:

Table A20 VMSs concentration on the anaerobic treatment, collected from the digester (ng/g dw).

Compound	16/05/2018	22/05/2018	29/05/2018	05/06/2018	12/06/2018
D3	105.1	204.3	59.6	214.70	142.18
L3	nd	nd	nd	nd	nd
D4	150.4	244.7	101.9	141.75	162.07
L4	8.3	16.4	7.5	10.07	11.73
D5	2265.3	4983.2	2286.9	2796.72	3127.69
L5	0.0	0.0	0.0	57.72	63.86
D6	718.0	1244.1	495.8	583.65	703.34
Total	**3247.0**	**6692.8**	**2951.7**	**3804.6**	**4210.9**

Table A21 VMSs concentration on the final sludge, dewatered sludge (ng/g dw).

Compound	16/05/2018	22/05/2018	29/05/2018	05/06/2018	12/06/2018
D3	nd	334.7	nd	nd	143.7
L3	nd	nd	nd	nd	nd
D4	211.8	208.9	91.0	51.2	90.0
L4	23.6	16.4	12.1	22.2	7.3
D5	5657.1	5033.9	2457.1	5702.9	1782.9
L5	140.7	138.3	55.9	132.9	40.0
D6	1608.5	1623.4	552.6	1230.5	428.7
Total	**7641.8**	**7355.6**	**2616.0**	**7139.6**	**2492.7**

Two samples were also extracted in our laboratory in order to assess differences between different types of sludge on VMSs concentrations. Results are shown in the Table A22.

Table A22 Concentrations of VMSs in different sludges from a WWTP from Aveiro (Portugal) and a WWTP from Chile (ng/g dw).

Compound	Aveiro	Chile
D3	277.4	63.6
L3	nd	nd
D4	166.3	30.1
L4	11.7	1.1
D5	3139.4	795.2
L5	71.9	7.7
D6	1558.5	259.7
Total	**5225.2**	**1157.3**