

Microalgal cultures for the bioremediation of urban wastewaters in the presence of siloxanes

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Abstract

Microalgae are widely used in the bioremediation of wastewaters due to their oxygenation potential, CO₂ fixation ability and efficient removal of various pollutants such as nitrogen, phosphorus, heavy metals, and contaminants of emerging concern (CECs). Siloxanes are chemically synthesised polymeric organosilicon molecules that are considered CECs. The global daily use of numerous industrial and consumer products containing these compounds leads to their constant presence in wastewater treatment plants (WWTPs). Since biogas is produced in WWTPs during anaerobic digestion, the presence of siloxanes in wastewaters results in the production of biogas enriched with these compounds. When biogas is combusted to generate energy, volatile methylsiloxanes (VMSs) are oxidised into microcrystalline silicon dioxide particles that cause the breakdown of, for instance, cogeneration equipment upon their deposition. The existing commercial technologies to remove siloxanes focus directly on biogas, having high limitations and costs. The biological removal of siloxanes from wastewaters instead could be an economical and more sustainable alternative. The present work aimed to evaluate microalgal bioremediation of urban wastewaters and determine if they can also remove VMSs, by growing the microalga *Chlorella vulgaris* in primary (PE) and secondary (SE) effluents. For this purpose, nitrogen, phosphorus, organic carbon and seven VMSs concentrations were evaluated over the course of each experiment. VMSs concentration was assessed in the following matrices: (i) air; (ii) microalgal biomass; and (iii) water (cells-free culture medium).

C. vulgaris grew successfully in both effluents, with identical specific growth rates: $0.036 \pm 0.002 \text{ d}^{-1}$ for the culture grown in the SE and $0.034 \pm 0.001 \text{ d}^{-1}$ for the PE. Approximately 52% of nitrogen and 87% of phosphorus were efficiently removed from the SE, due to microalgal assimilation. In the PE culture, 86% of nitrogen and 80% of phosphorus were removed, most likely due to the combined action of *C. vulgaris* with microorganisms present in the effluent. After 6 days of cultivation, there was a significant decrease in both dissolved organic and inorganic carbon. These results showed that microalgal cultures are a cost-effective and environment-friendly option for both secondary and tertiary wastewater treatments. In terms of VMSs removal, decamethylcyclopentasiloxane (D5) was the predominant VMS in both PE ($904 \pm 83 \text{ ng L}^{-1}$) and SE ($455 \pm 141 \text{ ng L}^{-1}$). A concentration decrease of approximately 98% was verified in the culture medium throughout the PE assay, most likely due to volatilisation. Three out of the seven analysed VMSs were detected in the biomass samples, but dodecamethylcyclohexasiloxane (D6) was the one that accumulated to a greater extent, since 48% of the initial mass of D6 was detected in the biomass samples while 22% remained in the aqueous phase. Although different experimental conditions must be tested to have definitive conclusions, the results are an indication of *C. vulgaris*' potential to remove specific VMSs from effluents, at least to a certain extent.

Keywords: Microalgae; Siloxanes; Wastewater bioremediation; Contaminants of emerging concern; Urban effluents; Nutrients removal.

Resumo

As microalgas são amplamente utilizadas na bioremediação de águas residuais devido ao seu potencial de oxigenação, capacidade de fixação de CO₂ e remoção eficiente de vários poluentes tais como azoto, fósforo, metais pesados e contaminantes emergentes (CEs). Os siloxanos são moléculas poliméricas de organo-silício sintetizadas quimicamente, consideradas CEs. A utilização diária de vários produtos industriais e de consumo que contêm estes compostos leva à sua presença constante em estações de tratamento de águas residuais (ETARs). Uma vez que o biogás é produzido em ETARs durante a digestão anaeróbia, da presença de siloxanos nas águas residuais resulta a produção de biogás com estes compostos. Quando o biogás sofre combustão para gerar energia, os metilsiloxanos voláteis (MSVs) são oxidados a partículas microcristalinas de dióxido de silício que provocam avarias, por exemplo, do equipamento de co-geração, aquando da sua deposição. As tecnologias comerciais existentes para remover siloxanos focam-se diretamente no biogás, tendo limitações e custos elevados, e a remoção biológica de siloxanos de águas residuais pode ser uma alternativa económica e mais sustentável. O presente trabalho visou avaliar a capacidade das microalgas na bioremediação de águas residuais urbanas, determinando se também conseguem remover MSVs através do cultivo da microalga *Chlorella vulgaris* em efluentes primários (EP) e secundários (ES). Para este fim, foram avaliadas, ao longo de cada experiência, concentrações de azoto, fósforo, carbono orgânico e de sete MSVs. As concentrações de MSVs foram avaliadas nas seguintes matrizes: (i) ar; (ii) biomassa; e (iii) água (meio de cultura sem células).

A microalga *C. vulgaris* cresceu com sucesso em ambos os efluentes, com taxas de crescimento específicas idênticas: $0,036 \pm 0,002 \text{ d}^{-1}$ para a cultura no ES e $0,034 \pm 0,001 \text{ d}^{-1}$ para o EP. Aproximadamente 52% do azoto e 87% do fósforo foram eficazmente removidos do ES, devido à assimilação por parte das microalgas. Na cultura EP, 86% do azoto e 80% do fósforo foram removidos, muito provavelmente devido à ação combinada da *C. vulgaris* com microrganismos presentes no efluente. Após 6 dias de cultivo, houve uma diminuição significativa tanto do carbono orgânico dissolvido como do inorgânico. Estes resultados mostraram que as culturas de microalgas são uma opção rentável e amiga do ambiente, tanto para os tratamentos secundários como terciários de águas residuais. Em termos de remoção de MSVs, o decametilciclopentasiloxano (D5) foi o MSV predominante tanto no EP ($904 \pm 83 \text{ ng L}^{-1}$) como no ES ($455 \pm 141 \text{ ng L}^{-1}$). Verificou-se uma diminuição na concentração de aproximadamente 98% no meio de cultura ao longo do ensaio EP, provavelmente devido à sua volatilização. Três dos sete MSVs analisados foram detetados nas amostras de biomassa, mas o dodecametilciclohexa-siloxano (D6) foi o que mais se acumulou, uma vez que 48% da massa inicial de D6 foi detetada nas amostras de biomassa enquanto que 22% se manteve na fase aquosa. Embora devam ser testadas diferentes condições experimentais, estes resultados são uma indicação do potencial da *C. vulgaris* para remover MSVs específicos de efluentes, pelo menos até certo ponto.

Palavras-chave: Microalgas; Siloxanos; Biorremediação de águas residuais; Contaminantes emergentes; Efluentes urbanos; Remoção de nutrientes.

Declaration

Declaro, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

Eva Salgado

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Nomenclature

Acronyms

Abs	Absorbance
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
ATP	Adenosine triphosphate
BBM	Bold's Basal Medium
BOD	Biochemical oxygen demand
BPA	Bisphenol A
BTF	Biotrickling filter
CCAP	Culture Collection of Algae and Protozoa
CEC	Contaminant of emerging concern
COD	Chemical oxygen demand
cVMS	Cyclic volatile methylsiloxane
C18	Octadecylsilane
C+	Positive control assay
C-PE	Negative control assay with PE
C-SE	Negative control assay with SE
DIC	Dissolved inorganic carbon
DOC	Dissolved organic carbon
DMSD	Dimethylsilanediol
dSPE	Dispersive solid-phase extraction
DW	Dry weight
D3	Hexamethylcyclotrisiloxane
D4	Octamethylcyclotetrasiloxane
D5	Decamethylcyclopentasiloxane
D6	Dodecamethylcyclohexasiloxane
D7	Tetradecamethylcycloheptasiloxane
ECHA	European Chemicals Agency
EDC	Endocrine-disrupting chemical
EPS	Extracellular polymeric substance
EU	European Union

GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
HRAP	High-rate algal pond
HS-SPME	Headspace-solid phase microextraction
HU	Hazen units
H ⁺ -NMR	Proton nuclear magnetic resonance
IS	Internal standard
IR	Infrared
LB	Laboratory blank
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
IVMS	Linear volatile methylsiloxane
L2	Hexamethylsiloxane
L3	Octamethyltrisiloxane
L4	Decamethyltetrasiloxane
L5	Dodecamethylpentasiloxane
M4Q	Tetrakis (trimethylsilyloxy)silane
na	Not applicable
nd	Not detected
NH ₄ -N	Ammonium nitrogen
NO ₃ -N	Nitrate-nitrogen
nr	Not reported
NTU	Nephelometric turbidity unit
OECD	Organisation for Economic Cooperation and Development
OD	Optical density
PAO	Polyphosphate accumulating organisms
PBR	Photobioreactor
PBT	Persistent, bioaccumulative and toxic
PDMS	Polydimethylsiloxane
PEMS	Polyethermethylsiloxane
PCPs	Personal care products
PO ₄ -P	Phosphate-phosphorus

PPCPs	Pharmaceuticals and personal care products
PSA	Primary and secondary amine
PE	Primary effluent
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RMSE	Root mean squared error
rpm	Rotations per minute
RRLC-MS	Rapid resolution liquid chromatography-tandem mass spectrometry
SB	Sample blank
SD	Standard deviation
SEAC	Committee for Socioeconomic Analysis
SPE	Solid-phase extraction
SE	Secondary effluent
TDC	Total dissolved carbon
THF	Tetrahydrofuran
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
TS	Total solids
TVS	Total volatile solids
UPLC-MS	Ultra-performance liquid chromatography-mass spectrometry
VMS	Volatile methylsiloxane
WW	Wet weight
vPvB	Very persistent and very bioaccumulative
WWTP	Wastewater treatment plant
¹³ C-NMR	Carbon-13 nuclear magnetic resonance

Notation

a	Slope of a calibration curve
$\frac{A_{VMS}}{A_{IS}}$	Ratio between the peak area for a specific VMS and the peak area for the IS
b	Intercept of a calibration curve
C_b	Individual VMS concentration in a lyophilised biomass sample (ng L ⁻¹)
C_{LSTD}	Lowest standard VMS concentration of a calibration curve (ng L ⁻¹)

$C_{s/b}$	Individual VMS concentration ($\text{ng g}_{\text{DW}}^{-1}$)
C_w	Individual VMS concentration in a water sample (ng L^{-1})
k	Nutrients uptake rate (d^{-1})
K_{oc}	Organic carbon-water partition coefficient
K_{ow}	Octanol-water partition coefficient
MR	Nutrient mass removal (mg L^{-1})
M_{w0}	Estimated mass of an individual VMS in the cells-free culture medium at t_0 (ng)
M_{wf}	Estimated mass of an individual VMS in the cells-free culture medium at t_f (ng)
M_b	Estimated mass of an individual VMS in the biomass from each culture at t_f (ng)
n	Data size
n_s	Number of standards
p	p -value
P_X	Biomass productivity ($\text{g}_{\text{DW}} \text{L}^{-1} \text{d}^{-1}$)
$P_{X,avg}$	Average biomass productivity ($\text{g}_{\text{DW}} \text{L}^{-1} \text{d}^{-1}$)
$P_{X,max}$	Maximum biomass productivity ($\text{g}_{\text{DW}} \text{L}^{-1} \text{d}^{-1}$)
RE	Nutrients removal efficiency (%)
R^2	Coefficient of determination
s_a	Standard deviation of the slope
s_b	Standard deviation of the intercept
S_f	Nutrient (N or P) concentration at t_f (mg L^{-1})
SNR_{LSTD}	Signal-to-noise ratio for the VMS standard with lower concentration
S_0	Nutrient (N or P) concentration at t_0 (mg L^{-1})
t_f	Time correspondent to the end of the experiment (d)
t_z	Time z correspondent to any experimental point (d)
t_{z+1}	Time correspondent to $t_z + 1$ (d)
t_0	Time correspondent to the beginning of the experiment (d)
t_1	Time correspondent to the beginning of the exponential growth phase (d)
t_2	Time correspondent to the end of the exponential growth phase (d)
V_{cs}	Volume of the concentrated sample (L)
V_e	Extract volume (L)
V_s	Sample volume (L)
X_f	Biomass concentration at t_f ($\text{g}_{\text{DW}} \text{L}^{-1}$)
X_z	Biomass concentration at t_z ($\text{g}_{\text{DW}} \text{L}^{-1}$)
X_{z+1}	Biomass concentration at t_{z+1} ($\text{g}_{\text{DW}} \text{L}^{-1}$)

X_0	Biomass concentration at t_0 ($\text{g}_{\text{DW}} \text{L}^{-1}$)
X_1	Biomass concentration at t_1 ($\text{g}_{\text{DW}} \text{L}^{-1}$)
X_2	Biomass concentration at t_2 ($\text{g}_{\text{DW}} \text{L}^{-1}$)
y	Experimental values used for the modified Gompertz model fits
$\hat{y}$	Predicted values by the modified Gompertz model
$\bar{y}$	Average of the experimental values y
λ	Lag time (d)
μ	Specific growth rate (d^{-1})

1. Introduction

1.1. Background and project presentation

The International Union of Pure and Applied Chemistry defines siloxanes as saturated silicon-oxygen hydrides with unbranched or branched chains of alternating silicon and oxygen atoms. Due to their antimicrobial properties, hydrophobicity and biocompatibility, siloxanes are used in various industrial applications, such as cosmetics, personal care products (PCPs), food industry, medicine, among others (Tansel & Surita, 2017). Even though these compounds have been around us for more than six decades, they are considered contaminants of emerging concern (CECs) (Rücker & Kümmerer, 2015). In wastewater treatment plants (WWTPs), their occurrence in wastewaters favours the partition to sludge, which leads to the production of biogas enriched with these compounds. When combusted to generate energy, siloxanes are oxidised into microcrystalline silicon dioxide and deposit on cogenerator parts, causing the breakdown of equipment and increasing the energy production costs (De Arespacochaga et al., 2015). Decamethylcyclopentasiloxane (D5) is usually found in large quantities in wastewaters and causes major problems. Moreover, it is one of the most commonly used volatile methylsiloxanes (VMSs) in PCPs (as a solvent or carrier), and it is released to the atmosphere in large quantities (McLachlan et al., 2010). Technologies to remove siloxanes from biogas already exist but have numerous limitations, such as the high implementation and operational costs. Biological removal of siloxanes from wastewaters instead of biogas could be an economical and environment-friendly alternative, contributing to the minimisation of soil and aquatic ecosystems contamination. Even though siloxanes removal and degradation studies have been conducted with bacteria, no investigation has been performed with microalgae.

Microalgae are a diverse group of microorganisms, comprised by eukaryotic microalgae and prokaryotic cyanobacteria, that conduct oxygen-evolving photosynthesis (Andersen, 2013). These microorganisms are used in a wide variety of applications, but one of the most remarkable is in wastewaters bioremediation, particularly in secondary and tertiary treatments of WWTPs. *Chlorella vulgaris*, a spherical unicellular eukaryotic green alga, is one of the most used microalgal species for this application, due to its tolerance to different environmental conditions and resistance to heavy metals and other potentially inhibitory compounds (Sydney et al., 2013). Even though several studies have been done on removing several contaminants from wastewaters using microalgae, no studies have been conducted to evaluate the growth of microalgae in environments with siloxanes and whether they can remove or degrade them. Accordingly, this project aimed to evaluate microalgal bioremediation of urban wastewaters and determine if they can also remove VMSs by growing the microalga *C. vulgaris* in urban wastewaters collected before and after the secondary treatment step.

1.2. Main objectives

The main objectives of this project were: **(i) to evaluate the growth of *C. vulgaris* in urban effluents:** study of the growth behaviour of this microalga in primary- and secondary-treated effluents collected from a Portuguese WWTP, by determining the biomass concentration in terms of dry weight over time, the specific growth rate and biomass productivity; **(ii) to investigate microalgal nutrients removal from the effluents:** time-course evaluation of the concentration of nitrogen, phosphorus, dissolved organic carbon (DOC), and chemical oxygen demand (COD) during *C. vulgaris* growth in the above-referred effluents; **(iii) to evaluate the microalgal removal of VMSs from the effluents:** analysis of the distribution of cyclic and linear VMSs over time in different matrices (air from the headspace of the bottles used for microalgal growth, microalgal biomass, and cells-free culture medium - water).

1.3. Thesis structure

The present document is divided into five chapters. Chapter 1 presents the context, main motivation, and scientific relevance of the work, as well as its main goals and contributions.

Chapter 2 presents a brief literature review, addressing: (i) the characteristics of siloxanes, applications and environmental emergence; (ii) the problems caused by these compounds in WWTPs, as well as existing biological and non-biological strategies to solve them; (iii) microalgal characteristics, cultivation and applications; (iv) the role of microalgae in wastewater treatment, with a particular emphasis on the removal of pollutants from real or synthetic wastewaters using *C. vulgaris*; and (v) the occurrence of siloxanes in algae.

Chapter 3 focuses on the methodologies used for: (i) cultivation of *C. vulgaris* in the selected effluents; (ii) physicochemical analysis of the effluents in terms of nitrogen, phosphorus, DOC, COD, colour and turbidity, total solids and total volatile solids; and (iii) VMSs quantification in different matrices.

Chapter 4 is dedicated to the results obtained regarding microalgal biomass production in the selected effluents and their role in nitrogen, phosphorus, organic carbon and VMSs removal, as well as the evaluation of siloxane distribution through the different studied matrices.

Chapter 5 presents the general concluding remarks, focusing on the accomplished objectives, limitations, and suggestions for future work, as well as a final global appreciation.

2. Literature review

2.1. Siloxanes

2.1.1. Definition and physicochemical properties

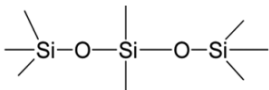
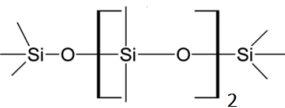
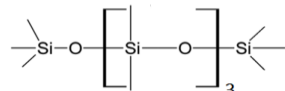
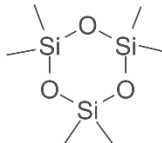
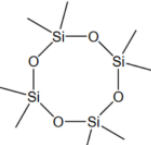
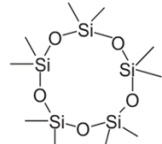
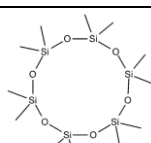
The term siloxane is a generic designation for a subgroup of silicones that contain silanol bonds, Si-O, with groups, such as methyl and ethyl, bonded to the silicon atom (Oshita et al., 2010). They can also be defined as chemically-synthesised polymeric organic silicon molecules that consist in a backbone of alternating Si-O units with organic sidechains attached to each silicon atom (Zhang et al., 2011). Therefore, their structure is described by the repeating group (R_2Si-O), which typically includes the organic substituents at the silicon atom. The Si-O bond is relatively long, about 1.64 Å, with large bond energy and very polarised, due to the low electronegativity of Si (Bletsou et al., 2013; Mojsiewicz-Pienkowska et al., 2016). In terms of spatial arrangement, these compounds can be classified as linear, branched, cyclic or cross-linked (Mojsiewicz-Pienkowska et al., 2016).

The unique chemistry of the Si-O bond and the type of organic substituents define their physicochemical properties (Mackay et al., 2015). Generally, siloxanes are compounds with low water solubility, low chemical reactivity, low surface tension and energy, high compressibility, low flammability, a smooth texture, and resistance to moisture, temperature, oxidation and UV radiation (Bletsou et al., 2013; Mojsiewicz-Pienkowska et al., 2016; Ryckebosch et al., 2011). They have an amphiphilic character, due to the presence of an inorganic chain with strong polar Si-O bonds on the one side, and non-polar organic groups bonded to the Si atom on the other. This characteristic allows their adhesion to human skin, by forming hydrogen bonds with lipids and proteins, making siloxanes a suitable component for skin care products (Mojsiewicz-Pienkowska et al., 2016).

Siloxanes can be subdivided into three major classes, depending on their molecular weight: volatile methylsiloxanes (VMSs), polydimethylsiloxanes (PDMSs) and polyethermethylsiloxanes (PEMSs) (Wang et al., 2013). VMSs are oligomeric alkylsiloxanes formed by repeating units of $(CH_3)_2SiO$ in either cyclic (cVMSs) or linear (lVMSs) configurations (Bletsou et al., 2013; Wang et al., 2013). The abbreviations D and L are used for cyclic and linear siloxanes, respectively, and the numbers after these letters refer to the number of silicon atoms in each molecule (Oshita et al., 2010). Some of the physicochemical properties of four cVMSs and three lVMSs, selected for the present work, are summarised in Table 1.

VMSs are volatile compounds with (i) low molecular weight; (ii) low water solubility that decreases with the increase of their chain length; (iii) relatively high vapour pressures; and (iv) low boiling points, therefore having a strong tendency to volatilise from aqueous solutions. A crucial characteristic that defines the environmental behaviour of these compounds is the partition coefficient between octanol and water, K_{ow} . This

Table 1 Summary of the physical and chemical properties of IVMSs and cVMSs

Compound Cas no. Chemical formula	Chemical structure	Water solubility at 23 °C ($\mu\text{g L}^{-1}$) ^a	Boiling point (°C) ^a	Vapour pressure at 25°C (mmHg) ^b	Log <i>K_{ow}</i> at 25 °C ^{c, d}	Molecular weight (g mol ⁻¹) ^e
Octamethyltrisiloxane (L3) 107-51-7 C ₈ H ₂₄ O ₂ Si ₃		0.0340	153	3.9	4.8	237
Decamethyltetrasiloxane (L4) 141-62-8 C ₁₀ H ₃₀ O ₃ Si ₄		0.00674	194	0.55	5.4	311
Dodecamethylpentasiloxane (L5) 141-63-9 C ₁₂ H ₃₆ O ₄ Si ₅		0.000309	232	0.070	6.0	385
Hexamethylcyclotrisiloxane (D3) 541-05-9 C ₆ H ₁₈ O ₃ Si ₃		1.560	135	10	3.85	222
Octamethylcyclotetrasiloxane (D4) 556-67-2 C ₈ H ₂₄ O ₄ Si ₄		0.056	176	1.3	4.45	297
Decamethylcyclopentasiloxane (D5) 541-02-6 C ₁₀ H ₃₀ O ₅ Si ₅		0.017	211	0.4	5.2	371
Dodecamethylcyclohexasiloxane (D6) 540-97-6 C ₁₂ H ₃₆ O ₆ Si ₆		0.005	245	0.02	5.86	445

^aDe Arespachochaga *et al.* (2015); ^bKim *et al.* (2013); ^cMeyers *et al.* (2013); ^dOshita *et al.* (2010); ^eBletsou *et al.* (2013); *K_{ow}*: octanol-water partition coefficient.

coefficient can be used to predict the environmental uptake of organic compounds by aquatic biota through sediment and water (Rücker & Kümmerer, 2015; Wang et al., 2013). It is also the most common way of expressing the lipophilicity of a compound, with higher values usually indicating stronger hydrophobicity, lower water solubility and higher adsorption of compounds onto the solid phase or the surface of microorganisms (Hena et al., 2021; Moldoveanu & David, 2015). VMSs have high K_{OW} values (>4) and, therefore, sorption by organic matter can compete with volatilisation as an additional transport mechanism (Kozerski et al., 2014). Another important parameter for quantifying the sorption behaviour of nonionic organic compounds in soil and sediment is the organic carbon-water partition coefficient, K_{OC} (Kozerski et al., 2014). For the seven VMSs described in Table 1, $\log(K_{OC})$ values between 4.34 and 7.36 have been reported (Kozerski et al., 2014; Panagopoulos et al., 2017). Decamethyltetrasiloxane (L4), dodecamethylpentasiloxane (L5) and dodecamethylcyclohexasiloxane (D6) have the highest values of K_{OW} and K_{OC} , lower water solubility and, consequently, a stronger tendency to adsorb to organic matter.

2.1.2. Applications and environmental concern

Due to their unique physicochemical properties, siloxanes are used in a wide variety of applications: (i) cosmetics, mainly in hair and skincare products, such as shampoos, creams and make-up; (ii) detergent and cleaning products, as foam inhibitors; (iii) medicinal products and devices, for instance, ointments for scars, emollients for skin conditions, implants and prosthesis; (iv) construction, as substances for the impregnation of paint; (v) various industrial processes, for example, as a replacement for organic solvents and as intermediates for the production of silicone polymers; and (vi) food, as a functional additive (Garcia et al., 2015; Mojsiewicz-Pieńkowska & Krenczkowska, 2018; Oshita et al., 2010).

According to different studies and publications, the world siloxanes production is estimated to be between 8 and 10 million tons each year (Mojsiewicz-Pieńkowska & Krenczkowska, 2018). As a result of their widespread use, it appears that these compounds are becoming an uncontrollable source of pollution. Consequently, siloxanes are currently considered CECs; initially, they were not considered as pollutants due to the lack of analytical data and methods, but nowadays, there is a growing concern about their persistence in the environment and toxic effects (Rücker & Kümmerer, 2015). CECs can be defined as synthetic or naturally-occurring chemicals or even microorganisms that are not usually monitored in the environment but have the capacity to enter the environment and originate known or suspected harmful ecological or human health impacts (Rosenfeld & Feng, 2011). Pharmaceuticals and personal care products (PPCPs), endocrine-disrupting chemicals (EDCs), pesticides, plasticisers and flame retardants are examples of CECs (Sutherland & Ralph, 2019). The presence of these compounds in the environment can be predominantly attributed to the discharge of effluents from WWTPs, that were not designed to specifically treat and remove CECs from wastewaters (Escapa et al., 2017). cVMSs, such as octamethylcyclotetrasiloxane (D4), decamethylcyclopentasiloxane (D5), and

dodecamethylcyclotetrasiloxane (D6), are the subject of considerable scientific interest and recent regulatory discussion and implementation, since they have been detected in water, air, sediments and biota (Mackay et al., 2015; Wang et al., 2013). D4, D5 and D6 are currently listed by the European Chemicals Agency (ECHA) as substances of very high concern due to their persistent, bioaccumulative and toxic (PBT), as well as very persistent and very bioaccumulative (vPvB), properties (European Chemicals Agency (ECHA), 2020). In fact, in 2016, the Committee for Socioeconomic Analysis of ECHA agreed on a restriction on the use of D4 and D5 in PCPs, such as shower gels and shaving foams. Furthermore, in 2018, the European Commission published the Regulation EC 2018/35 that restricts the use of D4 and D5 to concentrations lower than 0.1% by weight of either substance in wash-off PCPs from January 2020 (European Commission, 2018).

2.2. Siloxanes and wastewater treatment plants

2.2.1. Siloxanes occurrence in biogas

Due to the daily use of products containing siloxanes and their chemical derivatives, these pollutants are frequently found in WWTPs, predominantly D4 and D5, while the remaining siloxanes appear in lower amounts (Garcia et al., 2015; Shen et al., 2018). D5 is of particular interest due to its high consumer products usage (Mackay et al., 2015). The siloxanes route from wastewaters to wastewater treatment and biogas combustion is schematically represented in Figure 1. In WWTPs, over 50% of the VMs, in the forms of D4 and D5, volatilise mainly during the aeration phase of the treatment process, while about 17% remain in the input sludge of anaerobic digestion (Garcia et al., 2015). Since these compounds have low water solubility and high adsorption coefficients, they tend to be partially removed from the aqueous phase by adsorption in the particulate matter of sludge and sediment (Zhang et al., 2011). The sludge floc extracellular polymeric substances (EPSs) are believed to be the primary adsorbing sites for siloxanes (Soreanu et al., 2011).

In WWTPs, anaerobic digestion leads to biogas production, which is used as a renewable fuel in heat and electric power production through a cogeneration process (Garcia et al., 2015). Biogas is usually composed of CH₄ (40–75%), CO₂ (15–60%), and other pollutants at trace level concentrations: H₂S (0.005–2%), O₂ (0–1%), N₂ (0–2%), NH₃ (< 1%) and siloxanes (0–0.02%) (Ryckebosch et al., 2011). Studies have shown that the accumulation of siloxanes in sludge and their volatilisation leads to the production of biogas enriched with these volatile compounds with concentrations as high as 60 mg m⁻³ (Oshita et al., 2010; Soreanu et al., 2011). During biogas combustion, siloxanes are transformed into oxides that precipitate as glassy microcrystalline silica (SiO₂) and deposit on the surface of the engines, valves, spark plugs and cylinder heads, causing the breakdown of equipment, and, eventually, stopping the cogeneration process (Shen et al., 2018). These oxides are highly abrasive, wearing down the equipment, changing the combustion chamber in geometry, clogging lines, which

could cause accidental explosions, decreasing the system's useful lifetime and increasing operational and maintenance costs (Garcia et al., 2015; Shen et al., 2018). For this reason, manufacturers have defined limits for the concentration of VMs in gas-processing equipment. For instance, for internal combustion engines, depending on the manufacturer, the maximum values should be between 5-28 mg m⁻³ and, for microturbines, between 0.03-0.1 mg m⁻³ (Soreanu et al., 2011). Therefore, it is necessary to remove siloxanes upstream of the generating equipment to improve the cogeneration process.

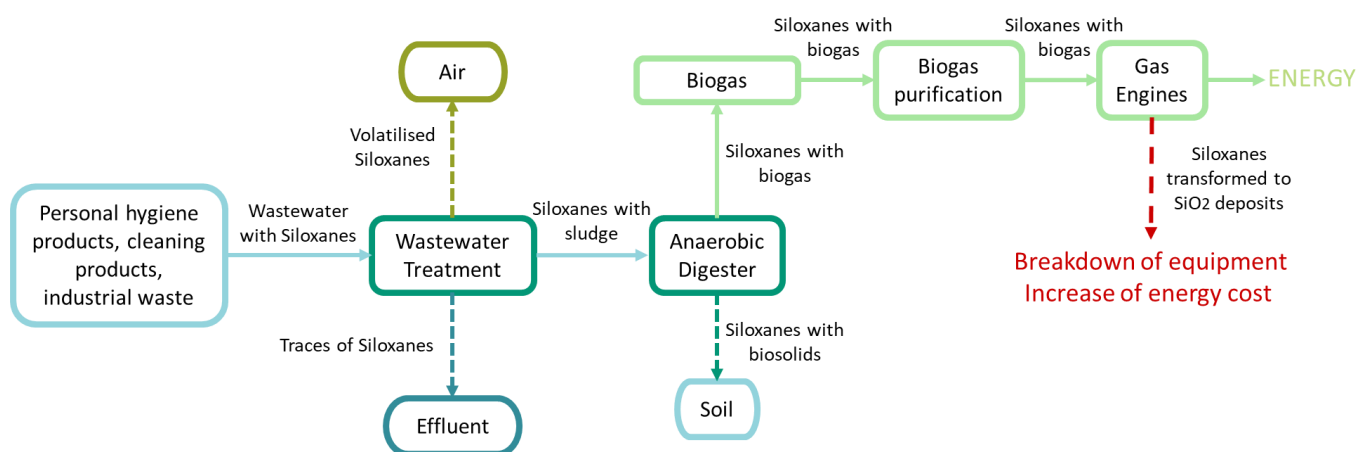


Figure 1 Siloxanes route from the source to wastewater treatment and biogas combustion, their occurrence in biogas and impact on the cogeneration process. Adapted from Soreanu et al. (2011).

Due to the adverse effects of VMs on the cogeneration process, many technologies and methods have been developed to remove them from biogas. Commercial applications include adsorption, absorption, and cryogenic condensation (Shen et al., 2018). At a smaller scale, biological removal techniques have been recently studied as an alternative.

2.2.2. Removal by non-biological processes

Adsorption is the primarily used technique for removing siloxanes from biogas, since it is a well-assessed, effective, easy-to-operate process, with wide and low-cost raw material sources that do not require extra chemical reagents (Shen et al., 2018). The most common method to achieve this removal is the adsorption with activated carbon, a porous non-polar adsorbent with a large surface area, which allows a siloxanes removal efficiency between 90-99% (Oshita et al., 2010; Shen et al., 2018). This technique requires two units or more, one for adsorption and another for adsorbent regeneration. However, siloxane polymerisation can cause the blockage of pores, which makes it impossible to regenerate the adsorbent effectively. This leads to the regular replacement of the adsorbent beds, significantly increasing the process costs (Gislon et al., 2013; Shen et al., 2018). Furthermore, factors such as the physical properties of the biogas, the type of activated carbon, the siloxane species, and water content in the biogas, have an impact on the adsorption capacity. If the relative humidity is high, the adsorption capacity decreases, which leads to the necessity of a pre-treatment step to remove the water through a refrigeration/condensation process (Shen et al., 2018). Other adsorbents such as

silica gel, molecular sieves or activated alumina generally require higher investment and operational costs, have lower removal efficiencies, larger consumption, and the low regeneration efficiency problem is maintained. Therefore, more research is needed to develop an easily recoverable and stable adsorbent with a high capacity to remove siloxanes (Shen et al., 2018).

Absorption is the second major process applied for siloxanes removal from biogas (Ruiling et al., 2017). Removal of siloxanes from biogas by absorption can be performed physically, using polar organic solvents to retain siloxanes, or chemically, employing concentrated acidic solutions to break Si–O bonds (Shen et al., 2018). Physical absorption's main limitations are the difficulty of completely removing siloxanes due to variations in concentrations of these compounds and the desorption of siloxanes, since highly volatile compounds are quickly stripped from the solvent at elevated gas flow rates (Ruiling et al., 2017). Besides its high implementation costs, chemical absorption implies the use of corrosive solutions, and, therefore, it is a dangerous technique, due to the potential health and environmental risks (Shen et al., 2018).

The removal of siloxanes by cryogenic condensation is performed by decreasing the biogas temperature until a condensate containing these compounds is formed in a refrigeration/condensation system. Depending on the siloxanes' volatility, temperatures can be decreased as far as to -70 °C (Ruiling et al., 2017). However, this technology alone cannot reach high siloxane removal rates, so it is usually applied together with other processes, such as adsorption. Furthermore, cryogenic condensation requires high investment and operational costs and increased energy demand (Shen et al., 2018).

2.2.3. Biological removal and degradation of siloxanes

Biological techniques to remove siloxanes are appealing alternative technologies that are often more convenient, economical and environment-friendly (Popat & Deshusses, 2008; Shen et al., 2018). Various microorganisms, such as bacteria from activated sludge, have proven to degrade certain siloxanes. As opposed to physical removal processes, in biological systems, siloxanes are typically degraded under catalytic conditions, depending on their environmental conditions. In biological degradation reactions, intermediates such as dimethylsilanediol (DMSD), silanetriols and formaldehyde are formed, and the final products are silicic acid, CO₂, and water. This reaction mechanism consists of the hydroxylation of a methyl group to a hydroxymethyl group, and successive oxidation to a formyl group (Soreanu et al., 2011). Table 2 presents a summary of a few studies on the biodegradation of siloxanes by bacteria.

In general, bioreactors (like bioscrubbers, biofilters, and biotrickling filters (BTF) inoculated with these cultures to remove siloxanes from gas streams) perform better than cultures in shake flasks, in terms of removal efficiency, due to the higher cell density achieved (Popat & Deshusses, 2008). Accettola *et al.* (2008) used bacteria isolated from activated sludge (mainly *Pseudomonas*) in an anaerobic BTF at laboratory scale, to remove D3 from an inlet air stream. The D3 removal efficiency was between 10–20%, due to mass transfer

Table 2 Summary of studies that revealed the ability of certain microorganisms to degrade cVMSs present in the culture medium as the only carbon source

Microorganisms	Studied siloxane	Culture conditions	Extraction and/or analysis methodology	Conclusions regarding siloxanes removal or degradation	Reference
<i>Bacillus subtilis</i> , <i>Proteus</i> species, <i>Pseudomonas aeruginosa</i>	D3 and D4 in liquid state	Batch cultures in glass test tubes, supplied with 0.2-0.5 mL of different siloxanes in 5 mL of culture medium	<u>D3, D4:</u> Liquid-liquid extraction with carbon tetrachloride followed by H ⁺ -NMR and ¹³ C-NMR spectrometry	•<i>Pseudomonas aeruginosa</i> degraded D3 and D4 (removal efficiency of 65%) •<i>Bacillus subtilis</i> and <i>Proteus</i> species degraded D4 (removal efficiencies of 35% and 42%, respectively)	Rościszewski <i>et al.</i> (1998)
Bacteria from activated sludge: <i>Rhodanobacter</i> , <i>Zooglea</i> , <i>Mesorhizobium</i> , <i>Xanthomonadacea</i> , and predominantly <i>Pseudomonas</i> sp.	D4 in liquid state	Aerobic batch culture in a shake flask, supplied with 4 mL of D4 in 150 mL mineral medium	<u>Degradation products:</u> H ⁺ -NMR and silicic acid test	•DMSD was at a higher concentration at the end of the experiment compared to the control group •D4 hydrolysis was favoured by bacteria, which means that biotic degradation seemed likely	Accettola <i>et al.</i> (2008)
Bacteria isolated from activated sludge	D4 in liquid state	•Aerobic batch culture in a shake flask, supplied with 500 mg L⁻¹ of D4 in mineral medium •Anaerobic batch culture in a shake flask, supplied with 500 mg L⁻¹ of D4 in mineral medium	nr	•Slow growth of the cultures on D4 as the sole carbon source and extremely slow batch biodegradation, even though microorganisms have an enormous potential to degrade siloxanes •Immobilised cell bioreactors inoculated with the same cultures performed better in D4 removal due to higher cell density	Popat & Deshusses (2008)
Microorganisms from activated sludge obtained from an anaerobic tank of a WWTP	D3, D4, D5 and D6 in liquid state	Anaerobic batch cultures in glass vials, supplied with three different concentrations of siloxanes (2, 5 and 10 µg L ⁻¹)	<u>DMSD:</u> SPE using THF and MgSO ₄ coupled with derivative GC-MS <u>D3, D4, D5, D6:</u> HS-SPME with polydimethylsiloxane/divinylbenzene fiber, followed by GC-MS	•After 60 h, D3 and D6 were only slightly biodegraded (3.0-18.1%), which may be due to their high volatility, while D4 and D5 were degraded about 44.4-62.8% •DMSD was found in the activated sludge-liquid mixture, and microbe catalysis hydrolysis was identified as one of the main degradation pathways for cyclic siloxanes in anaerobic tanks	Xu <i>et al.</i> (2013)

DMSD: dimethylsilanediol; GC-MS: gas chromatography-mass spectrometry; HS-SPME: headspace-solid phase microextraction; H⁺-NMR: proton nuclear magnetic resonance; IR: infrared; nr: not reported; SPE: solid-phase extraction; THF: tetrahydrofuran; WWTP: wastewater treatment plant; ¹³C -NMR: carbon-13 nuclear magnetic resonance.

limitations from the gaseous to the liquid phases. The authors concluded that further studies should be performed to improve the mass transfer, for instance, by using surfactants and/or structured packing, instead of random packing. Popat & Deshusses (2008) used bacteria isolated from activated sludge in both anaerobic and aerobic BTF at laboratory scale to remove D4 from a synthetic gas stream. They concluded that the removal efficiency in the anaerobic process (15%) was similar to the aerobic process (10%). These low removal efficiencies were attributed to the low water solubility of siloxanes and low mass transfer coefficients between the gas and the biological cell surface. Li *et al.* (2014) inoculated a laboratory-scale BTF with *Pseudomonas aeruginosa* to remove D4 from an inlet air stream and obtained a relatively high removal efficiency, over 74%. The authors concluded that the main reason for this increase in removal efficiency relative to previous studies was the biosurfactants produced by this microorganism, which were identified in the liquid phase of the BTF. In a recent study, Zhang *et al.* (2020) evaluated the removal of siloxanes from biogas using an anaerobic BTF inoculated with anaerobically-digested sludge and proposed a degradation pathway of cyclic D5 in the recycling liquid on the BTF. According to the authors, the degradation products were hydroxy end-blocked linear silanols, and cyclic and linear oligomers, mainly DMSD and hexamethylsiloxane (L2).

However, these bioreactors still have some limitations, such as the risk of fouling, blocking, and long-time operation process (Shen *et al.*, 2018). More studies are required to improve the mass transfer constraints and the removal efficiencies to implement this type of technology at a larger scale. Also, the biological removal of siloxanes from biogas using this type of bioreactors remains a costly alternative, which shows the necessity of studies on the adaptation of these principles to obtain effective biological technologies to remove these pollutants also directly from the wastewaters/sludge.

2.3. Microalgae

2.3.1. Definition and metabolism

Microalgae is a general term that refers to a diverse group of microorganisms that conduct oxygen-evolving photosynthesis, such as eukaryotic microalgae and prokaryotic cyanobacteria (blue-green algae) (Andersen, 2013). Over 25 000 microalgal species have been isolated and identified from both seawater and freshwater (Vale *et al.*, 2020). Microalgae are mainly photoautotrophs, using light as the energy source and CO₂ as a carbon source, converting it in adenosine triphosphate (ATP) and carbohydrates through photosynthesis (Umdu & Univ, 2020). However, in the presence of certain organic molecules and the absence of light, microalgae adopt a heterotrophic metabolism, using these molecules as a carbon source. Furthermore, mixotrophic metabolism is common in some of these microorganisms, in which CO₂ is needed for photosynthesis, the primary energy source, and the organic compounds are facultatively used for respiration.

This type of microalgae assimilates organic compounds as a carbon source and use inorganic compounds as electrons donors (Siqueira et al., 2018). Despite the metabolism adopted, their growth is dependent on six major elements that represent 99.9% of the microalgal biomass: carbon, oxygen, hydrogen, nitrogen, sulphur, and phosphorus (Umdu & Univ, 2020). For photoautotrophic growth, carbon is the most important element, with CO₂ being its primary source (Liu & Chen, 2016). Nitrogen and phosphorus are also very important, being considered two growth-limiting elements (Umdu & Univ, 2020). Nitrogen, a crucial element for regulating algal growth and metabolism, especially for protein synthesis, can be obtained from nitrate, ammonia, urea, glycine, and amino acids. On the other hand, phosphorus is an essential nutrient for the formation of nucleic acids, ATP (the energy form in cells) and the cell membrane, and its primary source for microalgae is phosphate, in the forms of H₂PO₄⁻, HPO₄²⁻ or PO₄³⁻ (Liu & Chen, 2016).

2.3.2. Cultivation

Compared to many types of plants, microalgae have high growth rates, high photosynthetic efficiency, remarkable adaptability, and are easy to cultivate (Escapa et al., 2017; Lv et al., 2017). According to Jegathese & Farid (2014), an ideal microalgae culturing system should provide effective illumination area, simple operation procedure, low contamination level, high land efficiency, optimal gas-liquid transfer, and low capital and production cost. Closed photobioreactors (PBRs) and open ponds are typical microalgae culturing systems. In closed PBRs, there is no direct exchange of material between the culture and the environment, which provides a highly controlled environment for the culture (Tan et al., 2020). There are three main configurations for these bioreactors: flat plate, horizontal-tubular, and vertical columns. All of them provide a lower risk of contamination than open ponds, higher photosynthetic efficiencies and minimisation of water losses through evaporation (Siqueira et al., 2018; Tan et al., 2020). However, they have high capital and operation costs, making them too expensive for large scale production (Jegathese & Farid, 2014). On the other hand, open ponds are simple and economic systems, having lower energy consumption, construction and operation costs, making them widely used industrially (Tan et al., 2020). There are various configurations for open ponds such as circular, thin-layer, multi-layer and raceway ponds (Costa et al., 2019). These are the most frequently used open pond types and the most economic bioreactor configuration for microalgae cultivation, consisting of a series of closed loops around central dividing walls that can contain baffles to enhance mixing (Jegathese & Farid, 2014; Posadas et al., 2015). However, compared to closed PBRs, open ponds require higher land areas, are more susceptible to contamination, and provide lower biomass productivities, mainly due to the lower mass transfer efficiencies achieved (Posadas et al., 2015; Siqueira et al., 2018).

2.3.3. Applications

Microalgae are a potential source of many interest compounds, such as polyunsaturated fatty acids, carotenoids, pigments, phycobiliproteins, polysaccharides, phycotoxins, lipids and proteins, that are used for

the production of biofuels, cosmetics, pharmaceuticals, human food and animal feed, among others (Chu, 2012; Rizwan et al., 2018). They are considered a prospective sustainable food source due to their rich biochemical composition, such as high protein content and high level of essential amino acids (Kusmayadi et al., 2021). Furthermore, they are a source of valuable nutrients, such as omega-3 and long-chain polyunsaturated fatty acids, and their pigments can act as antioxidants (Kusmayadi et al., 2021). Therefore, these organisms can be used as human food or animal feed, enhancing the nutritional content of conventional food preparations and positively affecting human and animal health (Spolaore et al., 2006). Extracts from certain microalgae from the genus *Arthrospira* and *Chlorella* are also used in the cosmetics market, particularly in skin and hair care products (Rizwan et al., 2018). For instance, *C. vulgaris* extracts are used as anti-ageing agents in some formulations, by stimulating collagen synthesis (Spolaore et al., 2006). Furthermore, microalgal products, such as Vitamin E and β -carotene, can be used as antioxidants, and polysaccharides, such as alginate and agar, as moisturising and thickening agents (Kumari et al., 2018). Besides the above-mentioned applications, microalgae are also a promising feedstock for biofuels, due to the fast-growing rate, high photosynthetic efficiency and high lipid content of some species (Peng et al., 2020). Biofuels have a higher degree of biodegradability and lower toxic emissions when compared to fossil-based fuels (Nascimento et al., 2014). Moreover, since microalgae can fix CO_2 photosynthetically, these microorganisms are used for the bio-mitigation of CO_2 emissions. According to Rizwan et al. (2018), if microalgae are used in biofuel production, the generation of CO_2 in power plants could be balanced by the fixation of CO_2 by these microorganisms, which would be a great environmental benefit. Lastly, one of the most remarkable applications of microalgae are in wastewater bioremediation and biomonitoring of environmental toxicants in WWTPs, discussed in detail in Section 2.4. Microalgae can remove nutrients, such as nitrogen and phosphorus, from wastewaters, and the nutrient-enriched biomass can be used to produce biofertilisers. Since phosphorus is vital for plant development and a limited non-renewable resource, recycling it in the form of biofertilisers and its return to the soil is a tremendous environmental benefit (Castro et al., 2020).

2.4. Microalgae in wastewater treatment

2.4.1. Microalgae in wastewater treatment plants

Wastewaters have considerable concentrations of nitrogen and phosphorus, essential nutrients for microalgal growth, making them a suitable medium for biomass production. Due to their oxygenation potential, efficient nutrients removal, and CO_2 mitigating ability, the use of plants or algae for wastewater treatment (phytoremediation) is an attractive option, since it is a cost-effective, eco-friendly technology that uses economically viable inputs to achieve a favourable commercial throughput (Posadas et al., 2015; Renuka et al.,

2013). Microalgae-based treatment systems can be more efficient than traditional systems, requiring lower capital investment and operation costs and providing natural disinfection (Sutherland & Ralph, 2019).

In WWTPs, wastewaters generally go through the following treatment steps: (i) preliminary treatment; (ii) primary treatment; (iii) secondary treatment; and (iv) tertiary treatment. During the secondary treatment step, since wastewaters have a great load of organic matter, heterotrophic bacteria are typically cultured in aerated tanks (e.g., activated sludge tanks) to oxidise organic matter into H_2O and CO_2 , using O_2 as an oxidising agent. The biochemical oxygen demand (BOD) is a measure of the respiratory demand of bacteria and other microorganisms during the oxidation of organic matter. Excessive BOD can lead to a decrease of dissolved oxygen in the receiving water bodies and the creation of anaerobic conditions that can be lethal to fish; therefore, secondary treatment aims at primarily reduce the organic load and consequently BOD (Abdel-Raouf et al., 2012). However, the traditional aeration entails large expenses, corresponding to 45–75% of the total operation costs in an activated sludge WWTP (Posadas et al., 2015). Alternatively, synergistic consortia between microalgae and bacteria have been studied and proven to be alternatives to the traditional secondary treatment process. By oxidising the organic matter, heterotrophic bacteria supply the CO_2 necessary for microalgae photosynthesis, as well as growth factors and vitamins, and, in turn, microalgae provide the O_2 required for bacteria (Bordel et al., 2009). Also, microalgae can facilitate bacterial growth through direct nutrients exchange or by providing nutrients from decomposing algal cells, and by protecting them from adverse environmental conditions (Unnithan et al., 2014). Therefore, using microalgae in this type of treatment reduces the operation costs in situations where oxygen must be provided externally and mechanically, and guarantees that an oxygenated effluent is discharged into the receiving water bodies (Bordel et al., 2009; Gonçalves et al., 2016).

The continuous discharge of effluents due to anthropogenic activities is the main cause of eutrophication, a process of changing the nutritional status of water bodies by increasing their nutrient sources, mainly nitrogen and phosphorus. This leads to harmful algal blooms that increase in geographic spread and frequency, causing oxygen depletion and the loss of numerous aquatic species, degrading aquatic ecosystems (Blackburn, 2013). Therefore, it is crucial to remove these nutrients from the effluents before releasing them into the water bodies. To reduce the risk of this type of events, nitrogen and phosphorus are removed from the secondary effluents through a tertiary treatment step, which usually comprises an anaerobic digestion process followed by nitrification and denitrification (Gonçalves et al., 2017). Even though this treatment can be performed chemically, the biological process is preferred due to its lower implementation costs and to the fact that it does not lead to secondary pollution (Abdel-Raouf et al., 2012). Nevertheless, the traditional biological method has some limitations: (i) low nutrients removal; (ii) the necessity of various cycles, tanks, and internal recycle of activated sludge, which results in high costs and energy requirements; and (iii) loss of potential valuable nutrients (Abdel-Raouf et al., 2012; Gonçalves et al., 2017; Posadas et al., 2015). Since microalgae require nitrogen and phosphorus for their growth, their use in the tertiary treatment of wastewaters is a cost-effective

approach to remove nutrients from wastewaters (Abdel-Raouf et al., 2012). Rapidly growing microalgae cultures can uptake these nutrients efficiently, resulting in valuable algal biomass. Besides nitrogen and phosphorus, microalgae can efficiently absorb or sequester heavy metals, common contaminants in wastewaters, making microalgal cultures a suitable and economical technology for removing these compounds in WWTPs (Abdel-Raouf et al., 2012).

2.4.2. Bioremediation using *Chlorella vulgaris*: removal of nutrients and emerging contaminants

One of the most remarkable microalgal species is *C. vulgaris*, a widely studied microalga, used in a broad variety of applications. *C. vulgaris* is a unicellular, non-mobile green algae, with spherical-shaped cells and a diameter ranging from 2 to 10 μm , that grows mainly in freshwater (Jo et al., 2020; Safi et al., 2014). *C. vulgaris* lacks flagella and reproduces asexually, forming daughter cells within the parental cells through mitosis (Jo et al., 2020). This microalga has a thick cell wall, which confers it high mechanical and chemical protection, a cytoplasm composed of minerals, water and soluble proteins, which holds the organelles, mitochondria, vacuoles, a single chloroplast, the Golgi body and a small nucleus (Safi et al., 2014; Sydney et al., 2013). Microalgae from the genus *Chlorella* can use organic carbon sources with (mixotrophic growth) or without (heterotrophic growth) CO_2 and light. *C. vulgaris* has been reported for both mixotrophic and heterotrophic growth, using glucose, glycerol and/or acetate as organic carbon sources in both cases (Liu & Chen, 2016).

Several studies have been conducted on the bioremediation of both synthetic and real wastewaters using various microalgal species, demonstrating their remarkable potential for this application. However, *C. vulgaris* is one of the most promising species due to its tolerance to different environmental conditions and resistance to heavy metals and other potentially inhibitory compounds (Sydney et al., 2013). Table 3 presents a summary of a few studies on real wastewater treatment using *C. vulgaris*, focusing on the removal of nutrients, such as nitrogen, phosphorus and carbon (in the forms of total organic carbon, TOC, and COD). Znad *et al.* (2018) showed that this microalga could efficiently remove nitrogen and phosphorus from both primary- and secondary-treated municipal effluents at different dilutions, with removal efficiencies over 80%. Furthermore, the specific growth rate increased with increasing effluent fractions, showing that culturing microalgae in municipal effluents can be a cost-effective approach for wastewater purification. Zhou *et al.* (2014) demonstrated that *C. vulgaris* could easily grow in a filtered municipal raw wastewater (influent) and not only provide very high nutrients removal efficiencies, 92% for total nitrogen (TN) and 82% for total phosphorus (TP), but also remove metals and organic contaminants. *C. vulgaris* has also demonstrated to adapt to highly concentrated municipal wastewater streams, generated by dewatering of sludge from primary- and secondary-settling (centrate), as Zhou *et al.* (2011) proposed. In this study, even though TN and TP removal efficiencies were lower than those mentioned before, one of the *C. vulgaris* strains used in these experiments showed

Table 3 Summary of a few studies on real wastewater treatment using *Chlorella vulgaris*, focusing on the removal of nitrogen (N), phosphorus (P), and organic matter (TOC and COD)

Type of wastewater	Parameter	Initial concentration	RE (%)	Growth parameters	Culture conditions	Cultivation period (days)	Reference
Highly concentrated municipal wastewater (centrate)	TN	134 mg L ⁻¹	> 50 *	μ : 0.293 d ⁻¹ $P_{X, avg}$: 0.121 g L ⁻¹ d ⁻¹	Batch cultures in flasks, with a light intensity of 50 μ mol photon m ⁻² s ⁻¹	3	Zhou <i>et al.</i> (2011)
	PO ₄ -P	212 mg L ⁻¹	> 65 *				
	TOC	960 mg L ⁻¹	> 82 *				
	COD	2324 mg L ⁻¹	> 74 *				
Filtered municipal raw wastewater (influent)	TN	6.64 mg L ⁻¹	92	μ : 0.36 d ⁻¹	Batch cultures in Erlenmeyer flasks, 25 °C, 150 rpm, 12 h light / 12 h dark regime, with a light intensity of 60 μ mol photon m ⁻² s ⁻¹	7	Zhou <i>et al.</i> (2014)
	TP	0.15 mg L ⁻¹	82				
Filtered primary wastewater	NH ₄ -N	40.8 mg L ⁻¹	61	μ : 0.61 d ⁻¹ $P_{X, avg}$: 0.09 g L ⁻¹ d ⁻¹	Batch cultures in Erlenmeyer flasks, 20 °C, continuously illuminated with a light intensity of 0.98 μ W cm ⁻²	28	AlMomani & Örmeci (2016)
	TP	10.0 mg L ⁻¹	35				
	COD	242 mg L ⁻¹	40				
Filtered secondary wastewater	NO ₃ -N	18.0 mg L ⁻¹	22	μ : 0.52 d ⁻¹ $P_{X, avg}$: 0.06 g L ⁻¹ d ⁻¹			
	TP	26.0 mg L ⁻¹	12				
	COD	59.0 mg L ⁻¹	49				
Urban wastewater at 30%	TN	59.31 mg L ⁻¹	88	μ : 1.06 d ⁻¹ $P_{X, avg}$: 0.19 g L ⁻¹ d ⁻¹	Batch cultures, 24 °C, 12 h light / 12 h dark regime with a light intensity of 40 μ mol photon m ⁻² s ⁻¹	10	Singh <i>et al.</i> (2017)
	TP	9.61 mg L ⁻¹	98				
Filtered primary domestic wastewater at 0.02% (v/v)	TN	2.7 mg L ⁻¹	85	μ : 0.30 d ⁻¹ $P_{X, avg}$: 0.041 g L ⁻¹ d ⁻¹	Batch cultures in Erlenmeyer flasks, 25-28 °C continuously illuminated with a light intensity of 60-70 μ mol photon m ⁻² s ⁻¹	12	Lam <i>et al.</i> (2017)
	TP	24.19 mg L ⁻¹	35				
Filtered secondary urban wastewater	NH ₄ -N	0.44 mg L ⁻¹	100	X_f : 1.167-1.575 g L ⁻¹	Batch cultures in PBRs, 24 °C, 12 h light / 12 h dark regime, with a light intensity of 180 μ mol photons m ⁻² s ⁻¹	21	Fernández-Linares <i>et al.</i> (2017)
	COD	38.50 mg L ⁻¹	100				
Filtered municipal primary wastewater	TN	30.6 mg L ⁻¹	100	μ : 1.2 d ⁻¹ X_{max} : 1.62 g L ⁻¹	Batch cultures in cylindrical glass columns, 24 °C, continuously illuminated with a light intensity of 180 μ mol photons m ⁻² s ⁻¹	13	Znad <i>et al.</i> (2018)
	TP	6.6 mg L ⁻¹	80				
Filtered municipal secondary wastewater	TN	11.8 mg L ⁻¹	83	μ : 0.62 d ⁻¹ X_{max} : 1.16 g L ⁻¹			
	TP	5.6 mg L ⁻¹	100				

*estimated values; COD: chemical oxygen demand; NH₄-N: ammonium-nitrogen; NO₃-N: nitrate-nitrogen; PBRs: Photobioreactors; PO₄-P: phosphate-phosphorus; $P_{X, avg}$: average biomass productivity; rpm: rotations per minute; TN: total nitrogen; TOC: total organic carbon; TP: total phosphorus; RE: removal efficiency; X_f : final biomass concentration; μ : specific growth rate.

promising TOC and COD removal efficiencies, over 82% and 74%, respectively. Besides municipal, urban or domestic wastewaters, several studies have shown the great bioremediation potential of this microalga on nutrients removal from hydroponic greenhouse cultivation wastewaters, piggery wastewaters, textile wastewaters, paper industry effluents, among others (Baglieri et al., 2016; Lim et al., 2010; Marjakangas et al., 2015; Silva et al., 2021).

Adding to inorganic pollutants like nitrogen, phosphorus and metals, wastewaters, particularly domestic, can contain organic pollutants, such as EDCs, PPCPs and pesticides, that nowadays are considered CECs (Zhou et al., 2014). Several studies have demonstrated that microalgal bioremediation of CECs involves essentially three removal mechanisms: bioadsorption, bioaccumulation and/or biodegradation. Bioadsorption is a passive process in which compounds are adsorbed to the cell wall components or to organic substances like EPSs (Sutherland & Ralph, 2019). In microalgal bioremediation, this is a species-specific process that occurs due to the hydrophobic or ionic interaction between the pollutants and the negatively charged microalgal cell wall, therefore depending on the hydrophobicity of the compounds (Hena et al., 2021). Bioaccumulation can be defined as the active transport of a pollutant through the cell wall into the cell and consequent binding to intracellular proteins (Hena et al., 2021; Sutherland & Ralph, 2019). Before a pollutant is bioaccumulated, bioadsorption must occur, but not all pollutants that are adsorbed onto the microalgal cell can be bioaccumulated (Hena et al., 2021). For instance, De Godos *et al.* (2012) showed that *C. vulgaris* could remove the antibiotic tetracycline by adsorption during synthetic wastewater treatment in a high-rate algae pond (HRAP), but no other mechanisms besides photodegradation were identified. Lastly, biodegradation is a metabolic breakdown of compounds into simpler molecules, either by catabolic enzymes or by its usage as a carbon source (Tiwari et al., 2017). In some cases, for the same pollutant, all these mechanisms can occur. Yu *et al.* (2017) evaluated the removal of the antibiotic ceftazidime by green algae. They demonstrated that algal removal of this contaminant could be separated into three steps: (i) rapid initial removal by adsorption, linked to physicochemical interactions between the contaminant and the cell wall; (ii) slow cell wall-transmission of molecules; and (iii) a final process of bioaccumulation and/or biodegradation.

Table 4 presents a summary of studies on the removal of CECs by *C. vulgaris* (from the culture medium or from synthetic/real wastewaters), as well as the correspondent biological removal mechanisms and CECs extraction and analysis methodologies. To evaluate the possible biodegradation of a specific CEC, some authors access their distribution in the following matrices: (i) CECs remaining in the culture medium; (ii) CECs adsorbed in the microalgal cell surface; (iii) CECs removed by abiotic processes such as photodegradation; and (iv) CECs accumulated in the algal biomass. Ji *et al.* (2014) used this methodology while studying the removal mechanisms of a hydrophobic EDC, bisphenol A (BPA), by cultivating *C. vulgaris* with different concentrations of this compound. The authors found that increasing BPA concentration reduced the growth rates and the uptake of nutrients such as nitrogen and phosphorus. Biodegradation and bioaccumulation of BPA were observed, and

Table 4 Summary of studies on the removal of organic contaminants of emerging concern by *Chlorella vulgaris* from synthetic and real wastewaters

Pollutant	Log K_{ow}	Initial pollutant concentration	RE (%)	Biological removal pathways	Culture conditions	Cultivation period (days)	Extraction and analysis methodologies	Reference
Tetracycline	nr	2 mg L ⁻¹ (daily)	69	Bioadsorption	Fed-batch cultures in an indoor HRAP, 10.0–17.5 °C, in synthetic wastewater, pH between 7.5–8.0, continuously illuminated at 10 W m ⁻² using daylight fluorescent and UV tubes	30	Injection of filtered samples into a HPLC system	De Godos <i>et al.</i> (2012)
Bisphenol A	3.42	1 mg L ⁻¹	38	Bioadsorption, bioaccumulation and biodegradation	Batch cultures in aluminium crimp-sealed serum bottles, 27 °C, in BBM, initial pH of 6.6, 150 rpm, 16 h light / 8 h dark regime, with a light intensity of 45-50 µmol m ⁻² s ⁻¹	10	Liquid-liquid extraction with dichloromethane:methanol (1:2 v/v) followed by SPE and UPLC-MS	Ji <i>et al.</i> (2014)
17 α -Boldenone	nr	0.0192 µg L ⁻¹	82	nr	Batch cultures in Erlenmeyer flasks, 25 °C, in filtered municipal raw wastewater, 12 h light / 12 h dark regime, 150 rpm, with a light intensity of 60 µmol photon m ⁻² s ⁻¹	7	SPE using sulphuric acid and methanol followed by RRLC-MS	Zhou <i>et al.</i> (2014)
17 β -Boldenone		0.0169 µg L ⁻¹	86					
Formestane		0.160 µg L ⁻¹	52					
Androstadienedione		25.6 µg L ⁻¹	89					
Bentazone		80.5 µg L ⁻¹	46					
Ciprofloxacin		0.0293 µg L ⁻¹	77					
Climbazole		0.147 µg L ⁻¹	30					
Clofibric acid		21.0 µg L ⁻¹	96					
Enrofloxacin		0.0262 µg L ⁻¹	77					
Erythromycin-H ₂ O		1.03 µg L ⁻¹	67					
Estrone		0.0917 µg L ⁻¹	87					
Fluconazole		0.044 µg L ⁻¹	27					
Lomefloxacin		0.0229 µg L ⁻¹	81					
Monensin		0.0180 µg L ⁻¹	80					
Narasin		0.0280 µg L ⁻¹	79					
Norfloxacin		0.0323 µg L ⁻¹	51					

Table 4 (continued)

Pollutant	Log <i>K_{ow}</i>	Initial pollutant concentration	RE (%)	Biological removal pathways	Culture conditions	Cultivation period (days)	Extraction and analysis methodologies	Reference
Pyrimethanil	2.84	4 mg L ⁻¹	>50	Bioadsorption	Batch cultures in flasks, 25-30 °C, in BG11 culture medium, pH of 9.5, 12 h light / 12 h dark regime with an irradiance of 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$	56	Liquid-Liquid extraction with hexane, vacuum evaporation, dissolution in acetonitrile followed by LC	Baglieri <i>et al.</i> (2016)
Metalaxyl	1.65		100	Biodegradation		14		
Fenhexamid	3.51		100	Biodegradation		49		
Diclofenac	nr	25 mg L ⁻¹	>69	Biodegradation	Batch cultures in bubble column PBRs, 25 °C, Mann and Myers medium, maintained at pH 7.5 by CO ₂ addition, 12 h light / 12 h dark regime with an irradiance of 370 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$	17	Centrifugation followed by the Injection of the supernatant into a HPLC system	Escapa <i>et al.</i> (2016, 2017)
Paracetamol			>21	Bioaccumulation		16		
Salicylic acid			>25	Bioaccumulation		16		
Levofloxacin	nr	1 mg L ⁻¹	12	Biodegradation and bioaccumulation	Batch cultures in flasks, 27 °C, in BBM, 150 rpm, 16 h light / 8 h dark regime, with a light intensity of 45-50 $\mu\text{mol m}^{-2} \text{s}^{-1}$	11	Liquid-liquid extraction using dichloromethane:methanol (1:2 v/v) followed by HPLC	Xiong <i>et al.</i> (2017)
Caffeine	-0.1	1 $\mu\text{g L}^{-1}$	31	Biodegradation	Batch cultures in flat panel PBRs, 20 °C, in BBM, maintained at pH 7.5 by CO ₂ addition, 12 h light / 12 h dark regime using white LED lights	12	Liquid-liquid extraction with methanol, mechanical extraction with glass beads, followed by LC-MS	Gojkovic <i>et al.</i> (2019)
Codeine	0.8		57	Biodegradation				
Diphenhydramine	3.3		98	Biodegradation				
Hydroxyzine	2.5		93	Biodegradation				
Memantine	3.4		100	Biodegradation				
Mirtazapine	2.9		69	Bioadsorption				
Oxazepam	2.5		10	Bioadsorption				
Metronidazole	-2.3	5 μM	100	Bioadsorption	Batch cultures in flasks, in BG11 culture medium, 25 °C, pH between 9-10, with an irradiance of 120 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$	18	Injection of filtered samples into a HPLC system	Hena <i>et al.</i> (2020)

BBM: Bold's Basal medium; HPLC: high performance liquid chromatography; HRAP: high-rate algae pond; LC: liquid chromatography; LC-MS: liquid chromatography-mass spectrometry; nr: not reported; PBRs: photobioreactors; RE: removal efficiency; rpm: rotations per minute; RRLC-MS: rapid resolution liquid chromatography-tandem mass spectrometry; SPE: solid phase extraction; UPLC-MS: ultra-performance liquid chromatography-mass spectrometry.

increased BPA concentrations led to increased accumulation, indicating an important role of bioaccumulation in the toxicity of this compound. Using similar methodologies for evaluating the removal mechanisms, Xiong *et al.* (2017) studied the removal of levofloxacin, a commonly discharged antibiotic in WWTPs. The authors found that by using strategies, such as increasing the salinity of the growth medium and acclimatising the microalgae through pre-exposition to this CEC, enhanced the removal efficiency to over 80%, mainly due to bioaccumulation and subsequent intracellular biodegradation by microalgae.

The microalgal ability to remove a pollutant from the culture medium or wastewater, as well as the type of mechanism used in this process, are related to the physicochemical properties of each compound. A very important parameter in this context is the pollutant's K_{OW} , since it reflects their adsorption tendency to organic matter such as the surface of microorganisms. A study conducted by Gojkovic *et al.* (2019), aiming to remove several active pharmaceutical ingredients (APIs) from the culture medium using *C. vulgaris*, showed significant removal efficiencies of up to 98% and 100% for diphenhydramine and memantine, respectively. The poor accumulation of these compounds in biomass samples suggested their metabolism by microalgae. Many other compounds were removed from the culture medium, and some were shown to accumulate in the algal biomass, such as mirtazapine and oxazepam. This study showed that *C. vulgaris* was highly efficient in removing APIs with $\log K_{OW} > 2.9$, and the authors suggested that this alga might have a specific detoxifying mechanism that degrades accumulated non-polar pharmaceuticals to less toxic intermediates. In general, lipophilic pharmaceuticals were removed efficiently but also accumulated in the algal biomass to a greater extent than polar APIs.

2.5. Siloxanes occurrence in algae: extraction and analysis

Studies have shown that VMSs can be found in marine microorganisms, such as macroalgae and phytoplankton. Table 5 summarises the concentrations of cVMSs determined in the few existent articles on these organisms, as well as the extraction procedures from biota samples. Sanchís *et al.* (2015) showed VMSs occurrence in soils, vegetation, phytoplankton and krill samples from the Antarctic Peninsula. Both cyclic and linear siloxanes were found in phytoplankton, but cVMSs at much higher concentrations, predominantly D3. A substantial negative correlation was found between total VMSs concentration in phytoplankton and sea surface salinity. According to the authors, once VMSs enter seawater, they immediately partition to phytoplankton due to their high hydrophobicity, which explains this correlation. And since VMSs are concentrated in phytoplankton, they can be transferred to upper levels of the food web. In terms of extraction and analysis of these compounds, the phytoplankton samples were lyophilised, and an ultrasound-assisted extraction procedure with hexane was performed, followed by a gas chromatography-mass spectrometry (GC-MS) analysis. Tetrakis (trimethylsilyloxy)silane (M4Q) was used as an internal standard (IS) to correct for the loss of

analyte during sample preparation or sample inlet. Jia *et al.* (2015) investigated the concentration and trophic magnification of methyl siloxanes in marine food webs from Northern China. Trophic magnifications of D4, D5, D6 and D7 were evaluated as the slope of lipid equivalent concentrations regressed against trophic levels of marine food web configurations. The green alga *Ulva lactuca* (commonly known as sea lettuce) was among the numerous marine species investigated in this study. The highest concentration found in this species, determined in terms of wet weight (WW), corresponds to D4, 2.21 ng g_{WW}⁻¹, and the lowest to D7, 0.81 ng g_{WW}⁻¹. In summary, the authors found a significant positive correlation between lipid normalised D5 concentrations and trophic levels in organisms, revealing the trophic magnification potential of this chemical in the marine food web. In this study, the siloxanes extraction procedure from the biota samples entailed homogenisation with a mixture of n-hexane and ethyl acetate, elution through a silica gel column, and solvent exchange followed by GC-MS analysis.

In a recent study performed by Rocha *et al.* (2019), three different species of algae (*Fucus vesiculosus*, *Porphyra* sp. and *U. lactuca*) were collected from different locations along the Atlantic coast of the north and centre of Portugal to determine the presence of siloxanes. Siloxanes were detected in most analysed macroalgae samples, and there was a strong predominance of cVMSs over linear ones, with D5 and D6 found at higher concentrations. Table 5 presents the maximum and minimum detected values of cVMSs in algal biomass. The "Quick, Easy, Cheap, Effective, Rugged, and Safe" (QuEChERS) extraction and clean-up technique was used, followed by GC-MS quantification. QuEChERS is a user-friendly solvent and time-saving approach that comprises two steps: (i) salting-out extraction using an organic solvent, in which the aqueous phase is separated from the organic phase; and (ii) clean-up involving a dispersive solid-phase extraction (d-SPE) using different porous sorbents and/or salts to remove interfering substances (Perestrelo *et al.*, 2019).

Table 5 Summary of siloxane concentrations already determined in algal biomass

Matrix	Siloxane extraction and analysis procedure	Siloxane concentration					Reference
		D3	D4	D5	D6	D7	
<i>Ulva lactuca</i>	1. Addition of 100 ng of M4Q (IS) to the samples and stabilisation for 30 min; 2. Shaking with 5 mL n-hexane:ethyl acetate (1:1 v/v) for 30 min and centrifugation for 5 min at 1 000 g. This step was repeated 3 times; 3. The supernatants were combined, and rotary evaporated to 1 mL; 4. The 1-mL extracts were passed through a silica-gel column, eluted with hexane:dicloromethane (1:1 v/v), and rotary evaporated to 1 mL; 5. Solvent-exchange into isooctane, reduced to 1 mL under nitrogen, and analysis by GC-MS.	nr	2.21 ng g _{WW} ⁻¹	1.23 ng g _{WW} ⁻¹	1.11 ng g _{WW} ⁻¹	0.81 ng g _{WW} ⁻¹	Jia <i>et al.</i> (2015)
Phytoplankton	1. Lyophilisation of the samples; 2. Spiking with surrogates: 30 µL of m-xylene-d ₁₀ and naphthalene-d ₈ 10 ng µL ⁻¹ and stabilisation for 3 h at 4 °C; 3. Addition of 6 mL of hexane followed by an ultrasonic bath for 25 min and centrifugation for 10 min at 1 700 g; 4. Collection of 1 mL of the supernatant, addition of M4Q at 10 ng µL ⁻¹ as IS, and analysis by GC-MS.	4.22 ng g _{DW} ⁻¹	0.93 ng g _{DW} ⁻¹	3.24 ng g _{DW} ⁻¹	1.17 ng g _{DW} ⁻¹	-	Sanchís <i>et al.</i> (2015)
<i>Ulva lactuca</i>	1. Spiking 2.5 g of finely cut samples with M4Q at 15 ng g ⁻¹ ; 2. Addition of 10 mL of n-hexane, 3 min of vortex and ultrasonication for 15 min;	0.22-0.70 ng g _{DW} ⁻¹	2.47-2.74 ng g _{DW} ⁻¹	0.62-7.37 ng g _{DW} ⁻¹	1.13-2.80 ng g _{DW} ⁻¹	-	Rocha <i>et al.</i> (2019)
<i>Porphyra</i> sp.	3. Addition of 6 g of anhydrous MgSO ₄ and 1.5 g of CH ₃ COONa, vortex for 3 min and centrifugation for 10 min at 2 670 g; 4. Transfer of the supernatant to a tube containing 900 mg of MgSO ₄ , 300 mg of PSA-bonded silica and 150 mg of C18;	0.49-1.63 ng g _{DW} ⁻¹	0.22-5 ng g _{DW} ⁻¹	0.28-12.01 ng g _{DW} ⁻¹	1.25-33.34 ng g _{DW} ⁻¹	-	
<i>Fucus vesiculosus</i>	5. Vortex and centrifugation as before, supernatant reduced to 1.5 mL under nitrogen, and analysis by GC-MS.	0.27-2.67 ng g _{DW} ⁻¹	0.12-1.89 ng g _{DW} ⁻¹	0.20-26.99 ng g _{DW} ⁻¹	0.29-5.11 ng g _{DW} ⁻¹	-	

C18: Octadecylsilane; DW: dry weight; D3: hexamethylcyclotrisiloxane; D4: octamethylcyclotetrasiloxane; D5: decamethylcyclopentasiloxane; D6: dodecamethylcyclohexasiloxane; D7: tetradecamethyl-cycloheptasiloxane; GC-MS: Gas chromatography-mass spectrometry; IS: internal standard; M4Q: tetrakis (trimethylsilyloxy)silane; nr: not reported; WW: wet weight.

3. Technical description

3.1. Chemicals and materials

3.1.1. Urban effluents

The effluents used in this study were collected from a Portuguese WWTP with 159 000 equivalent-inhabitants. The wastewater treatment process in this WWTP, presented in Figure 2, consists of: (i) a preliminary treatment, in which solids, coarse elements, sand and grease are removed by filtration through bar screens and physical separation in aerated grit-grease removers; (ii) a primary treatment, where primary sludge is separated from grease and oils in large sedimentation tanks; (iii) a secondary or biological treatment, in which dissolved and suspended organic matter is significantly reduced in aeration tanks or biological reactors. From this WWTP, two types of effluents were collected: one after the secondary treatment step, secondary effluent (SE), and another after the primary treatment step, primary effluent (PE). Effluent samples were stored at -20 °C until the effluent characterisation was performed.

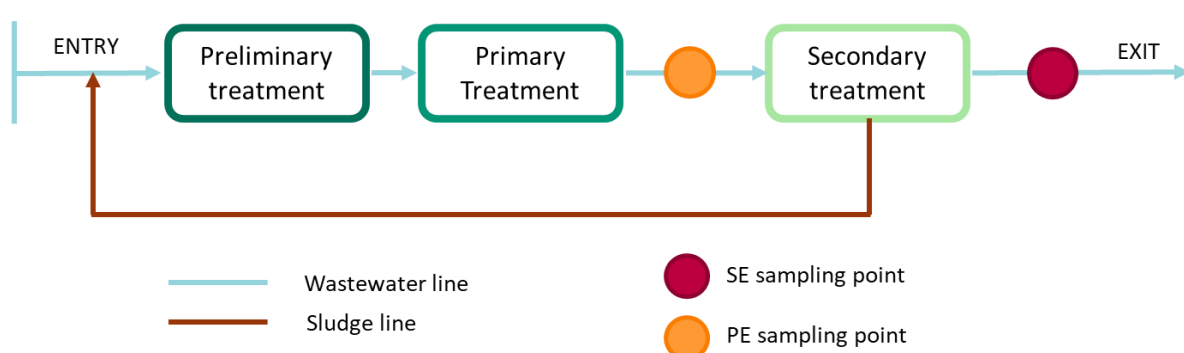


Figure 2 Scheme of the treatment process and effluent sampling points in the WWTP selected for this study.

3.1.2. Microalgae and culture medium

The microalga used in this project, *C. vulgaris* CCAP 211/11B, was obtained from the Culture Collection of Algae and Protozoa (CCAP, Oban, United Kingdom). Stock solutions were prepared in 100-mL Erlenmeyer flasks, with a working volume of 50 mL, using the modified OECD (Organisation for Economic Cooperation and Development) test medium, composed by four distinct solutions (A, B, C and D, see detailed composition in Appendix I), and distilled water. Erlenmeyer flasks were maintained at room temperature under a continuous light supply of approximately $6.50 \mu\text{mol m}^{-2} \text{s}^{-1}$. Agitation was promoted through an Unimax 1010 orbital shaker (Heidolph, Germany), set at 100 rotations per minute (rpm). After 30 days of cultivation, the stock solutions were transferred to five 500-mL glass bottles that were maintained under the same light and temperature conditions. After 15 days, cells grown in the 500-mL bottles were harvested through centrifugation at 12 000 rpm (22 114 g), 20 °C, for 10 minutes in an Avanti J-25 centrifuge (Beckman, Brea, CA, USA).

3.1.3. Chemicals for siloxane analyses

Seven VMSs, D3, D4, D5, D6, L3, L4 and L5, were analysed in the present study and M4Q was used as the IS. Individual VMSs standards and M4Q with a purity level higher than 97% were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual 1.0 g L⁻¹ stock solutions of these standards in *n*-hexane and then a final 5 mg L⁻¹ stock mixture were prepared, to be used in the construction of the calibration curves. The 5 mg L⁻¹ M4Q standards used in the samples extraction procedures were prepared in analytical grade *n*-hexane or acetone, obtained from VWR (Fontenay-sous-Bois, France), and protected from light in amber glass vials. All preparations were kept in the dark at -20 °C until use. Specifically for the QuEChERS procedure, the following reagents were used: ethyl acetate and dichloromethane from VWR (Fontenay-sous-Bois, France), anhydrous magnesium sulphate from Panreac AppliChem (Barcelona, Spain), primary and secondary amine (PSA) and octadecylsilane (C18) from Supelco (Bellefonte, PA, USA). Nitrogen for sample evaporation with a purity of 99.9999% was provided by Air Liquide (Maia, Portugal).

3.2. Effluent characterisation

Before its usage for microalgal cultivation, the effluents were allowed to settle overnight and then filtered through GF/A grade glass microfibre filters (Whatman, Maidstone, United Kingdom), using a vacuum filtration system. The physicochemical characteristics of both raw and filtered SE and PE are presented in Table 6. The methodology used to characterise these effluents was the following: (i) pH and conductivity were determined using Consort's C6010 electrochemical analyser (Brussels, Belgium); (ii) colour was determined in Hazen units, as described in the Portuguese definitive norm NP-627:1972 (NP-627, 1972); (iii) turbidity was measured using a Hanna Instruments HI88703 turbidimeter (Rhode Island, USA); (iv) COD, TP, total solids (TS) and total volatile solids (TVS) were determined according to the Standard Methods for the Examination of Water and Wastewater, through the 5220-D, 4500-P E, 2540 B and 2540 E tests, respectively (APHA, 2017); (v) total dissolved carbon (TDC), dissolved organic carbon (DOC), dissolved inorganic carbon (DIC) and total nitrogen (TN) were determined by filtrating the effluent using 0.22-µm cellulose acetate membranes (Orange Scientific, Braine-l'Alleud, Belgium) and analysing the filtrate in an organic carbon analyser (TOC-VCSN, Shimadzu); (vi) Nitrate-nitrogen (NO₃-N) concentration was determined using the United States Environmental Protection Agency (USEPA)-approved Brucine Colorimetric Method (US EPA, 1971); and (vii) Phosphate-phosphorus (PO₄-P) was quantified using the ascorbic acid colorimetric method, as described by Lee *et al.* (2009). The calibration curves data for each of these determinations are presented in Table II.1 of Appendix II. VMSs levels in the effluents were determined as described in Section 3.6. The raw PE revealed much higher colour, turbidity, DOC, COD, TS and TVS values than the raw SE, in which the organic matter was in its oxidised form due to the biological oxidation step. Nitrogen and phosphorus in the SE were mainly in the oxidised form, which explains

the similarity between TN and TP values with the NO₃-N and PO₄-P levels, respectively. In the PE, even though P was in the oxidised form, N was still in the reduced form, as it did not go through oxidation. For this reason, NO₃-N was not detected in this effluent.

Table 6 Physicochemical characterisation of the raw and filtered effluents used in this study

Parameters	Value				Unit
	Raw SE	Filtered SE	Raw PE	Filtered PE	
pH	7.1	7.7	7.3	8.1	-
Conductivity	1 822	nd	4 250	nd	µS m ⁻¹
Colour	43 ± 1	29 ± 7	260 ± 7	76 ± 8	HU
Turbidity	2.4 ± 0.2	0.8 ± 0.2	55 ± 3	5.5 ± 0.4	NTU
Chemical oxygen demand (COD)	24 ± 2	23 ± 4	76 ± 2	87 ± 4	mg O ₂ L ⁻¹
Dissolved organic carbon (DOC)	31 ± 26	10 ± 2	200 ± 37	63 ± 11	mg C L ⁻¹
Total dissolved carbon (TDC)	51 ± 26	26 ± 5	202 ± 37	99 ± 11	mg C L ⁻¹
Dissolved inorganic carbon (DIC)	19.66 ± 0.01	16 ± 3	0.74 ± 0.01	35.6 ± 0.2	mg C L ⁻¹
Total nitrogen (TN)	15 ± 1	14 ± 4	25.89 ± 0.02	25 ± 6	mg N L ⁻¹
Nitrate-nitrogen (NO ₃ -N)	12.25 ± 0.08	10.67 ± 0.02	<LOD	<LOD	mg N L ⁻¹
Total phosphorus (TP)	1.63 ± 0.02	nd	2.21 ± 0.05	nd	mg P L ⁻¹
Phosphate-phosphorus (PO ₄ -P)	2.83 ± 0.01	2.4 ± 0.3	3.0 ± 0.2	3.0 ± 0.3	mg P L ⁻¹
Molar N:P ratio	10	13	26	18	-
Total solids (TS)	1.118 ± 0.004	nd	1.47 ± 0.02	nd	g L ⁻¹
Total volatile solids (TVS)	0.143 ± 0.004	nd	0.22 ± 0.01	nd	g L ⁻¹
Hexamethylcyclotrisiloxane (D3)	127 ± 35	142 ± 63	184 ± 88	147 ± 26	ng D3 L ⁻¹
Octamethylcyclotetrasiloxane (D4)	246 ± 16	201 ± 99	198 ± 85	168 ± 78	ng D4 L ⁻¹
Decamethylcyclopentasiloxane (D5)	455 ± 141	376 ± 115	904 ± 83	954 ± 92	ng D5 L ⁻¹
Dodecamethylcyclohexasiloxane (D6)	233 ± 39	197 ± 88	247 ± 21	142 ± 22	ng D6 L ⁻¹
Octamethyltrisiloxane (L3)	<LOD	<LOD	<LOD	<LOD	ng L3 L ⁻¹
Decamethyltetrasiloxane (L4)	<LOD	<LOD	<LOD	<LOD	ng L4 L ⁻¹
Dodecamethylpentasiloxane (L5)	1.7 ± 0.7	<LOD	16 ± 22	<LOD	ng L5 L ⁻¹

HU: Hazen units; PE: primary effluent; LOD: limit of detection; nd: not determined; N:P ratio: nitrogen to phosphorus ratio; NTU: Nephelometric turbidity unit; SE: secondary effluent.

3.3. Experimental setup

The experiments regarding microalgal growth in previously filtered SE and PE were conducted in batch mode using 5-L bottles as a cultivation system. The following experimental conditions were tested: (i) two positive control bottles (C+) containing the *C. vulgaris* inoculum with 4.5 L of the modified OECD test medium; (ii) two negative control bottles (C-SE) containing only 4.5 L of filtered SE; (iii) two bottles with the *C. vulgaris* inoculum and 4.5 L of filtered SE (SE); (iv) two negative control bottles (C-PE) containing only 4.5 L of filtered PE, one exposed to light and another wrapped in aluminium paper to eliminate the possibility of siloxanes removal by photodegradation; and (v) two bottles with the *C. vulgaris* inoculum and 4.5 L of filtered PE (PE). The C+, SE and PE bottles were inoculated with the previously harvested biomass pellets, giving an initial

average biomass concentration of approximately $1 \text{ g}_{\text{DW}} \text{ L}^{-1}$. The cells were cultured until the stationary growth phase was reached. Therefore, the experiments had a duration of 7 d, for the SE and C-SE bottles, and 9 d, for bottles C+, C-PE and PE, at room temperature, $21 \pm 1 \text{ }^{\circ}\text{C}$ under continuous light (provided by an LED panel) with an average intensity of $42 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$. Agitation and CO_2 supplementation were performed by bubbling atmospheric air, filtered through $0.22\text{-}\mu\text{m}$ cellulose acetate membranes (Orange Scientific, Braine-l'Alleud, Belgium), at a flow rate of 1.5 L min^{-1} , using AP-180 air pumps from Trixie (Flensburg, Germany). The experimental setup is presented in Figure 3.

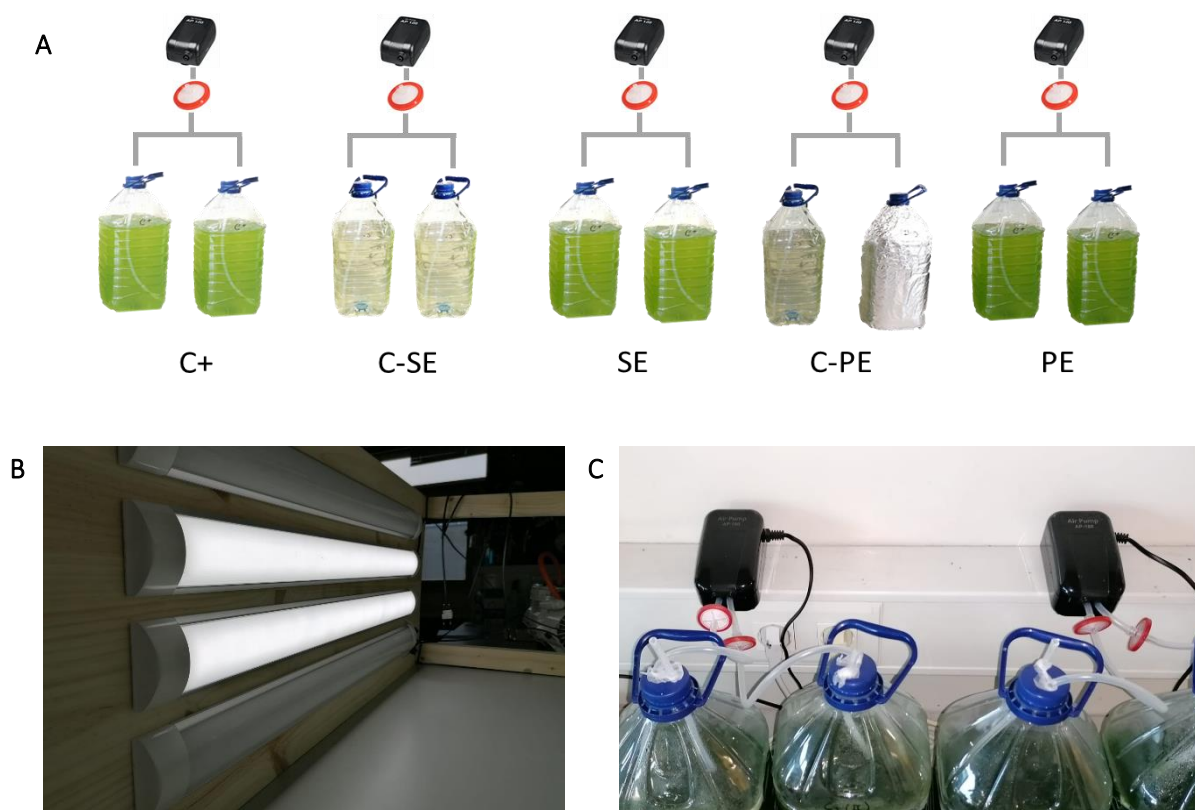


Figure 3 Experimental setup: (A) Schematic representation of the microalgal cultivation setup; (B) LED panel; (C) Close-up of the aeration system.

Regarding the cultures' monitoring, temperature and pH of the cultures were evaluated daily, using a Consort's C6010 electrochemical analyser (Brussels, Belgium), and light intensity was measured on the first and last days of each experiment, using a portable photo/radiometer (Delta OHM HD2102.2). To ensure that the experimental conditions were similar in each bottle, the pH of the microalgal cultures was adjusted daily using a 10% (v/v) HCl solution, to match the value of the negative controls. Furthermore, the colour and turbidity of the cells-free culture media were determined on the first and last days of each experiment. Table III.1 of Appendix III presents a summary of the samples collected from all the bottles for each assay, as well as the collection days and sample volume.

3.4. Microalgal growth monitoring in urban effluents

To evaluate *C. vulgaris* growth, 5-mL samples were collected once a day to measure the culture's optical density (OD) at 680 nm, using a V-530 UV/VIS spectrophotometer from Jasco. To build a calibration curve between OD and biomass concentration in terms of dry weight ($\text{g}_{\text{DW}} \text{L}^{-1}$), that gathers data from all microalgal cultures, 25-mL samples were collected. The samples were inserted in previously dried and weighed porcelain crucibles, 10 mL for each sample (in duplicate), and dried for 24 h at 105 °C. After this period, the crucibles were allowed to cool to room temperature in a desiccator and weighed. The concentration of biomass in terms of dry weight corresponds to the mass loss in the drying process, divided by the sample volume.

The specific growth rate, μ (d^{-1}), in the exponential growth phase was calculated according to Liang *et al.* (2013), using Equation 1, where X_2 and X_1 correspond to the biomass concentration, in $\text{g}_{\text{DW}} \text{L}^{-1}$, at times t_2 and t_1 , in days, which correspond to the end and beginning of the exponential growth phase, respectively.

$$\mu = \frac{\ln(X_2 / X_1)}{(t_2 - t_1)} \quad (1)$$

Biomass productivities, P_X , were determined as described in Equation 2, for each pair of consecutive points, where X_z corresponds to the biomass concentration at time t_z and X_{z+1} represents the biomass concentration at time t_{z+1} . Maximum biomass productivities, $P_{X, \text{max}}$ ($\text{g}_{\text{DW}} \text{L}^{-1} \text{d}^{-1}$), correspond to the maximum values determined by this equation. The average biomass productivities, $P_{X, \text{avg}}$ ($\text{g}_{\text{DW}} \text{L}^{-1} \text{d}^{-1}$) were determined as indicated in Equation 3, where X_f and X_0 correspond to the biomass concentration, in $\text{g}_{\text{DW}} \text{L}^{-1}$, at times t_f and t_0 , in days, which correspond to the end and the beginning of each experiment, respectively.

$$P_X = \frac{X_{z+1} - X_z}{t_{z+1} - t_z} \quad (2)$$

$$P_{X, \text{avg}} = \frac{X_f - X_0}{t_f - t_0} \quad (3)$$

On the last day of the experiment, the remaining culture from each bottle was centrifuged at 12 000 rpm (22 114 *g*), 20 °C, for 10 minutes (using a Beckman Avanti J-25 centrifuge), and the pellet/biomass was frozen at -80 °C, lyophilised, and stored in amber glass flasks until further analysis. These samples were used to determine the siloxanes concentration in the biomass, as described in Section 3.6.

3.5. Nutrients removal

To assess nutrients removal, nitrogen, phosphorus, COD, DOC, DIC and TDC levels were determined within the cultivation time, according to the methodologies described in Section 3.2. While in assays C+, C-SE and SE, nitrogen concentration was determined based on $\text{NO}_3\text{-N}$, for experiments C-PE and PE, the

measurements were performed directly in the organic carbon analyser, since N was in its reduced form in the assays using the PE. Phosphorus concentration over time was determined based on the PO₄-P levels, since it was in its oxidised form in all the experimental conditions. Samples were collected in duplicate from each bottle, centrifuged at 4 000 rpm (1 707 *g*) for 10 min, at 20 °C, using an Eppendorf 5804 R centrifuge (Hamburg, Germany), and the supernatant was frozen at -20 °C until further analysis. DOC, DIC, TDC and COD samples were collected with less frequency in assays C-SE and SE, since the organic load was much lower in these experiments compared to the assays with PE. Mass removal of nitrogen and phosphorus, *MR*, was calculated according to Equation 4, where *S*₀ corresponds to nutrients concentration at the beginning of each experiment, in mg L⁻¹, and *S*_f corresponds to the final nutrients concentration, in mg L⁻¹. Nutrients removal efficiencies (*RE*) were determined as indicated in Equation 5.

$$MR = S_0 - S_f \quad (4)$$

$$RE (\%) = \frac{S_0 - S_f}{S_0} \times 100 \quad (5)$$

The experimental data regarding nitrogen and phosphorus removal over time was fitted to the modified Gompertz model, as represented in Equation 6, where *k* is the nutrients uptake rate (d⁻¹) and *λ* is the lag time (d) (Zwietering et al., 1990). These kinetic parameters were calculated by minimising the sum of squared residuals using the Solver supplement of Microsoft Excel. The quality of the models was evaluated by calculating the coefficient of determination (*R*²), and the root mean squared error (RMSE). RMSE and *R*² were calculated according to Equations 7 and 8, where the variable *y* corresponds to the experimental values, *ŷ* to the predicted model values, *ȳ* to the average experimental values and *n* to the data size. The closer the parameters *R*² and RMSE are to 1 and 0, respectively, the higher the quality of the model fit.

$$S(t) = S_i + (S_f - S_i) \times \exp \left[- \exp \left[k (\lambda - t) + 1 \right] \right] \quad (6)$$

$$R^2 = 1 - \frac{\sum_{i=1}^n (y_i - \hat{y}_i)^2}{\sum_{i=1}^n (y_i - \bar{y})^2} \quad (7)$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^n (y_i - \hat{y}_i)^2}{n}} \quad (8)$$

3.6. Siloxanes extraction and quantification

3.6.1. Air samples

Air samples were collected from the headspace of the bottles on the first and last days of each experiment, using 1-L Tedlar air sampling bags from Supelco. A gas chromatography – ion mobility spectrometry device (GC-IMS-SILOX) from GAS (Dortmund, Germany) was used to quantify the concentrations of various

individual VMSs (L3, L4, L5, D3, D4 and D5). This device provides very similar results between duplicates; therefore, only a single measurement was performed for each sample.

3.6.2. Water samples

Throughout each experiment, 70-mL samples were collected and centrifuged at 4 000 rpm (1 707 *g*), for 10 min, at 20 °C, using an Eppendorf 5804 R centrifuge. The supernatant (water or cells-free culture medium) was collected and frozen at -20 °C until further analysis. To extract the siloxanes from the water samples, a liquid-liquid extraction procedure was performed in duplicate. Firstly, 30 mL of each sample and 25 µL of a 5 mg L⁻¹ M4Q standard in acetone (IS) were transferred to a 50-mL polypropylene conical bottom centrifuge tube. The samples were homogenised and settled for 30 min. Then, 10 mL of hexane was added, and the mixture was vortexed, sonicated in a 720 W JP Selecta ultrasonic bath (Barcelona, Spain) for 10 min, and centrifuged for 5 min at 4 000 rpm (2 760 *g*) using a Hettich KG D-78532 centrifuge (Tuttlingen, Germany). The organic phase (supernatant) was transferred to a 12-mL amber glass vial. The sample volume was reduced under a gentle nitrogen stream to a volume of 0.5 mL and stored in an amber glass vial at -20 °C until further analysis. Laboratory blanks (LBs) were prepared following the same procedure but without the sample addition step.

3.6.3. Microalgal biomass samples

To evaluate the siloxane concentration in the lyophilised biomass samples, a QuEChERS extraction was performed in duplicate. Due to limitations in the lyophilised biomass obtained from each experiment, in the first step of the QuEChERS extraction procedure, approximately 1 g of biomass for the SE assay and 0.7 g for assays C+ and PE, were weighed into a 50-mL polypropylene conical bottom centrifuge tube, designated QuEChERS 1. This tube contained 2.5 g of anhydrous MgSO₄ to remove water residues, spiked with 100 µL of a 5 mg L⁻¹ solution of M4Q in hexane. After the mixture was vortexed, the first extraction step was performed by adding 5 mL of hexane, followed by vortex, sonication (using a 720 W JP Selecta ultrasonic bath) for 10 min, and centrifugation for 5 min at 4 000 rpm (2 760 *g*) using a Hettich KG D-78532 centrifuge. The organic layer (supernatant) was collected and transferred to the second tube, designated QuEChERS 2, which contained 300 mg of MgSO₄, 300 mg of PSA-bonded silica and 50 mg of C18. The second and third extraction steps were performed by adding 5 mL of hexane:dichloromethane (1:1) and 5 mL of hexane:ethyl acetate (1:1) to the pellet, respectively, both followed by vortexing, sonication for 10 min, and centrifugation for 5 min at 4 000 rpm (2 760 *g*). After this step, the supernatant was also transferred to the QuEChERS 2 tube. The last step consisted of a dispersive solid-phase clean-up step to remove undesired compounds. The QuEChERS 2 tube containing the organic layer was vortexed, centrifuged for 5 min at 4 000 rpm (2 760 *g*), and the supernatant was transferred to a 12-mL amber glass vial. The sample volume was reduced to 1 mL under a gentle nitrogen stream and stored in an amber glass vial at -20 °C until further analysis. LBs were prepared following the same procedure but without the addition of the microalgal biomass.

3.6.4. GC analyses for siloxanes quantification

The siloxanes quantifications in water and biomass samples were performed by a researcher of the LEPABE group using a Varian Ion Trap GC–MS system (Walnut Creek, CA, USA), equipped with a 4000-GC gas chromatograph, a 240-MS ion trap mass spectrometer, a CP-1177 split/splitless injector and a CP-8410 auto-sampler. The injector was adapted with a Merlin microseal system, as opposed to the typical silicone rubber septa, to avoid contamination. Compound separation was accomplished in a low-bleed Agilent DB-5 ms ultra-inert column (30 m length $\times$ 0.25 mm diameter, 0.12 μ m film thickness) at a constant flow of helium (1.0 mL min⁻¹). This type of column avoids bleeding (natural degradation of the stationary phase) and minimises background VMS contamination. The following temperature program was used: 35 °C held for 5 min, raised at 10 °C min⁻¹ to 95 °C, then 5 °C min⁻¹ to 140 °C min⁻¹ and lastly 35 °C min⁻¹ to 300 °C (held for 5.43 min), in a total runtime of 30 min. The 1 μ L injection volume was in split mode, with a split ratio of 100. Temperatures of manifold, ion trap, transfer line, and injector were 50, 200, 250 and 200 °C, respectively. The mass spectrometer was operated in the electron ionisation mode (70 eV), and for quantitative analysis of target compounds, time-scheduled selected ion storage mode was applied. The filament emission current was 10 μ A. This equipment was used to analyse the following VMSs: D3, D4, D5, D6, L3, L4 and L5. The equations used to calculate siloxane concentration, as well as the calibration curves corresponding to each siloxane for both water and biomass samples, are presented in Appendix IV. It is important to note that to avoid external siloxane contamination, this work was developed in an environment in which PCPs were avoided by all members of the laboratory, detergents with siloxanes were not used, nitrile gloves were worn at all times, all the non-graduated glass material was rinsed with distilled water and acetone, and baked at 400 °C before usage.

3.7. Statistical analysis

Average and standard deviation (SD) values were determined for each parameter. To evaluate statistically significant differences between each experiment, the Student's paired *t*-test was used. For the kinetic parameters from the modified Gompertz models, a one-way analysis of variance (ANOVA) was used to analyse differences among the mean values. Statistical tests were performed at a significance level of 0.05.

4. Results and discussion

4.1. Microalgae growth monitoring in urban effluents

The pH and temperature variations over time can be found in Figure III.1, Appendix III. Over the course of the experiment, the temperature values remained constant and near 21 °C, meaning that the light supplied to the cultures did not affect the temperature in the bottles. The initial pH values were identical between the cultures grown in effluents and the respective negative controls, meaning that the addition of the inoculum did not affect the initial pH of the cultures. However, the pH values of the negative control bottles were higher than the respective effluents. This increase might be justified by the removal of organic matter during the filtration process, which altered some of the effluent's physicochemical characteristics. Around day 2, the pH of the microalgal cultures started to increase and, therefore, pH adjustments had to be done throughout each experiment. This is a common occurrence in microalgae cultures, since, during photosynthesis, these microorganisms consume CO₂, which accumulates mainly as HCO₃⁻. Steady-state usage of HCO₃⁻ as the original carbon source for photosynthesis leads to the accumulation of OH⁻ in the cell. Therefore, to neutralise these ions, there is an increase in H⁺ uptake from the culture medium, which leads to the rise in its pH (Chi et al., 2011).

Figure III.2 from Appendix III presents the OD values over time for each experiment. The OD of the microalgal cultures gradually increased throughout the assays, but it remained close to zero for the negative control bottles, since there was no microalgal growth in these experiments. The data of the calibration curve that plots the OD of the microalgal cultures at 680 nm against biomass concentration in g_{DW} L⁻¹, is presented in Table II.1 of Appendix II. Although the method used to determine biomass concentration in terms of dry weight typically leads to highly variable results, a good adjustment to the experimental data was obtained. Using the data from the calibration curve and the OD values obtained for each culture, growth curves were built for *C. vulgaris*, represented in Figure 4. Overall, the results show that this microalga grew successfully in all the experimental conditions. The microalgal lag phase was non-existent in all experiments, and by days 6, 7 and 8, cultures SE, PE, and C+, respectively, were no longer in the exponential growth phase, and the deceleration or stationary phase was reached. The differences in the duration of the exponential growth phase are related to nitrogen and phosphorus availability in each experiment: higher nutrients concentration leads to longer exponential growth phases.

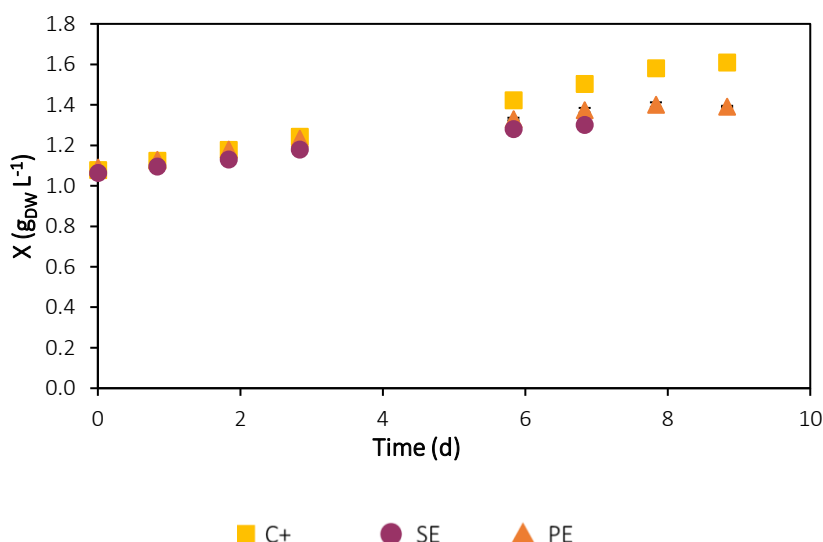


Figure 4 *Chlorella vulgaris* growth curves obtained for the C+, PE, and SE assays. Error bars correspond to the standard deviation of the mean obtained from two independent experiments.

Table 7 shows the growth parameters determined for *C. vulgaris* in each experiment. The specific growth rates were not statistically different ($p > 0.05$) between the assays SE, $0.036 \pm 0.002 \text{ d}^{-1}$, and PE, $0.034 \pm 0.001 \text{ d}^{-1}$, but were significantly lower ($p < 0.05$) than C+, $0.0472 \pm 0.0005 \text{ d}^{-1}$. The same correlation was found for the average/maximum biomass productivities, $0.035 \pm 0.003/0.050 \pm 0.009 \text{ g}_{\text{DW}} \text{ L}^{-1} \text{ d}^{-1}$ and $0.034 \pm 0.001/0.054 \pm 0.003 \text{ g}_{\text{DW}} \text{ L}^{-1} \text{ d}^{-1}$ for assays with SE and PE, respectively, and $0.060 \pm 0.002/0.08 \pm 0.02 \text{ g}_{\text{DW}} \text{ L}^{-1} \text{ d}^{-1}$ for C+. The only growth parameter in which assays PE and SE showed a significant difference was the maximum biomass concentration. In the experiment with PE, a statistically higher ($p < 0.05$) maximum biomass concentration was reached, $1.40 \pm 0.01 \text{ g}_{\text{DW}} \text{ L}^{-1}$, compared to the assay with SE, $1.30 \pm 0.02 \text{ g}_{\text{DW}} \text{ L}^{-1}$. This difference was most likely due to the higher nutrients concentration of the PE, since higher nitrogen and phosphorus availability leads to faster and higher microalgal growth. Overall, the higher growth parameters for the positive control are explained by the optimal and adequate composition of the modified OECD culture medium for microalgal growth and higher nutrients concentration, compared to the urban effluents. Furthermore, the effluents are likely to contain potentially inhibitory compounds for microalgal growth. The specific growth rates in this study were lower than the ones obtained by Znad *et al.* (2018) when growing *C. vulgaris* supplied with a light intensity of $180 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in municipal secondary (0.62 d^{-1}) and primary effluents (1.2 d^{-1}) with similar nutrients concentrations. This difference could be explained by a higher inhibitory effect of the effluents used in the present study and differences in the culturing conditions, such as the lower light intensity ($42 \mu\text{mol m}^{-2} \text{ s}^{-1}$), resulting in lower photosynthetic efficiency. These results were also lower than those reported by AlMomani & Örmeci (2016) in a similar experiment, 0.61 d^{-1} for a primary effluent and 0.52 d^{-1} for a secondary effluent. However, the initial biomass concentrations were much lower compared to this study, and the cultivation time much higher (28 d), which could explain these discrepancies.

Table 7 Growth parameters determined for *Chlorella vulgaris* in each experiment

Experiment	μ (d ⁻¹)	X_{max} (g _{DW} L ⁻¹)	$P_{X, max}$ (g _{DW} L ⁻¹ d ⁻¹)	$P_{X, avg}$ (g _{DW} L ⁻¹ d ⁻¹)
C+	0.0472 ± 0.0005 ^a	1.61 ± 0.01 ^a	0.080 ± 0.002 ^a	0.060 ± 0.002 ^a
SE	0.036 ± 0.002 ^b	1.30 ± 0.02 ^b	0.050 ± 0.009 ^b	0.035 ± 0.003 ^b
PE	0.034 ± 0.001 ^b	1.40 ± 0.01 ^c	0.054 ± 0.003 ^b	0.034 ± 0.001 ^b

$P_{X, avg}$: average biomass productivity; $P_{X, max}$: maximum biomass productivity; X_{max} : maximum biomass concentration; μ : specific growth rate. Values are presented as the mean ± standard deviation obtained from two independent experiments. Different letters (a, b, c) within the same column represent statistically different values ($p < 0.05$).

Wastewaters usually have high turbidity and colour due to the presence of highly diffusive particulate matter and coloured substances (Martínez et al., 2018). This causes a limitation in the light path in PBRs, decreasing photosynthetic photon flux density and reducing the photosynthetic efficiency (Valev et al., 2020). Consequently, a reduced biomass production and microalgal growth are verified. Table III.2 of Appendix III presents the colour and turbidity measurements of the cells-free culture media at the beginning and end of each experiment. Compared to raw effluents, the lower values in the filtered effluents show that the filtration process led to a significant decrease in colour and turbidity. On the one hand, the modified OECD medium showed lower turbidity compared to the filtered PE, but identical to the filtered SE. Overall, the turbidity values in the filtered effluents were low and since the growth was very similar between the SE and PE assays, this parameter does not appear to have influenced microalgal growth. On the other hand, the filtered effluent's colour could have affected microalgal growth. The colour was much higher in the effluents than the OECD medium, in which the values were below the LOQ. Therefore, the photosynthetic efficiency was most likely higher in the C+ assay, contributing to the higher growth in this experiment. Despite these expected growth differences, the results show that *C. vulgaris* grew successfully in the urban effluents and that they constitute a suitable culture medium for this microalga.

Analysing the colour and turbidity variations from the beginning to the end of each experiment, it is possible to observe that in the C-SE assay, turbidity and colour remained constant, while in the C-PE there was a statistically significant decrease ($p < 0.05$) in both parameters. This decrease might have been due to other microorganisms in the PE, which consumed some of the organic matter (as discussed in Section 4.2.) and possibly coloured substances, reducing colour and turbidity. In the SE assay, the turbidity remained constant. There was a statistically significant increase in colour ($p < 0.05$), which could have been due to a few microalgal cells in the samples owed to a poor separation between the pellet and the supernatant. In the PE assay, the colour remained constant ($p > 0.05$), and turbidity decreased significantly ($p < 0.05$), most likely as a result of the removal of organic matter by the combined action of *C. vulgaris* and the microorganisms present in the effluent.

4.2. Nutrients removal

4.2.1. Nitrogen and phosphorus

According to the European Union (EU) directives 1991/271/EEC and 1998/15/EC, for WWTPs with over 100 thousand population-equivalent, which is the case of the WWTP selected for this study, the limits for nutrients concentration in discharged effluents are: (i) 10 mg N L⁻¹ for TN or a minimum percentage of reduction of 70-80%; (ii) 1 mg P L⁻¹ for TP or a minimum reduction percentage of 80%; and (iii) 125 mg O₂ L⁻¹ for COD or a minimum reduction percentage of 75% (Directive 1991/271/EEC, 1991; Directive 1998/15/EC, 1998). Taking this legislation into account, the potential of *C. vulgaris* to remove nitrogen and phosphorus from the urban effluents and reduce the nutrients levels to values below these legislation limits was evaluated. Figure 5 presents the time-course evolution of nitrogen and phosphorus concentration in each experiment, and the model fits of the modified Gompertz model to the experimental data. Table 8 presents the initial nutrients concentrations, removal parameters, and kinetic parameters determined through the modified Gompertz model. The results showed that when cultured in urban effluents, *C. vulgaris* can efficiently remove nitrogen and phosphorus, reaching values below the EU legislation limits within the cultivation time. Overall, the modified Gompertz model appears to reflect a good adjustment to the experimental data, since RMSE values were equal to or below 1.265 and R² values were equal to or over 0.945. In both effluents, the molar N:P ratios were within the optimal range (5 to 30) for microalgal growth (Choi & Lee, 2015). Despite small variations, the nutrients concentrations in the filtered effluents were in accordance with results obtained for the raw effluents and, therefore, the filtration process does not appear to have affected these parameters. The initial nitrogen and phosphorus concentrations were not significantly different ($p > 0.05$) between the effluent-grown cultures and the respective negative controls; hence the addition of the inoculum did not have an impact on these parameters.

Initial nitrogen concentration was statistically higher ($p < 0.05$) in the modified OECD medium than in the filtered effluents. However, the difference was mild compared to the PE but nearly four times higher than in the SE. In the C-SE assay (Figure 5 A), nitrogen concentration remained approximately constant over the course of the experiment and, therefore, no removal was observed. The lowest mass removal (5.66 ± 0.01 mg L⁻¹) and lowest culture time necessary to reach the legislation limit (2 d) were observed for the SE assay (Figure 5 A), which can be explained by the lower initial nitrogen concentration. In the C-PE assay, a nitrogen mass removal of 11.06 ± 0.02 mg L⁻¹ was observed, which might have been due to the action of microorganisms present in the effluent. However, after 9 days, nitrogen concentration was still over the legislation limit (Figure 5 C). A significantly higher ($p < 0.05$) mass removal was observed in the PE assay compared to the remaining experiments, 28 ± 1 mg L⁻¹. Comparing these results with the C-PE experiment, there was a significant improvement in nitrogen removal when *C. vulgaris* was present since the mass removal nearly tripled in these

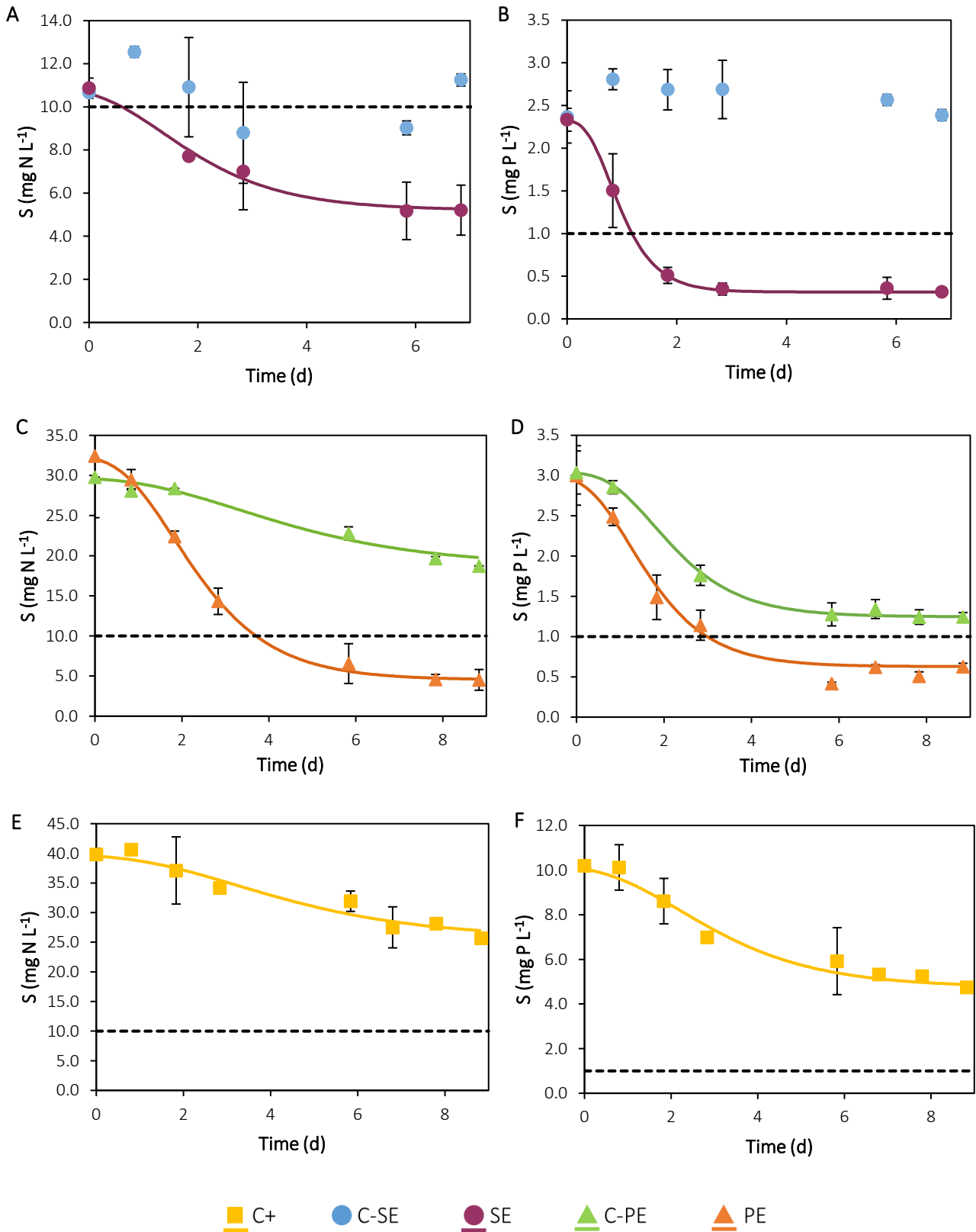


Figure 5 Time-course evolution of nitrogen (A, C and E) and phosphorus (B, D and F) concentration in assays C-SE/SE (A and B), C-PE/PE (C and D) and C+ (E and F). Error bars correspond to the standard deviation of the mean obtained from two independent experiments. The filled lines represent the model fit of the modified Gompertz model to the experimental data. The dashed lines correspond to the EU legislation limits in discharged effluents: 10 mg N L⁻¹ and 1 mg P L⁻¹.

conditions. Furthermore, after 6 days, the concentration levels were below the legislation limit, as shown in Figure 5 C. Comparing the mass removal in the C-PE assay with the C+ assay ($14.1 \pm 0.3 \text{ mg L}^{-1}$), the sum of these results is in accordance with the mass removal in the PE assay. This correlation indicates that the improvement in the PE compared to the C-PE resulted from the combined action between *C. vulgaris* and the microorganisms in the effluent. In terms of kinetic parameters for nitrogen removal, the mean uptake rates and lag times were not statistically different ($p > 0.05$) between all the experiments, according to the ANOVA statistical test.

Table 8 Nitrogen and phosphorus initial concentration, removal parameters, and kinetic parameters correspondent to the modified Gompertz model obtained for each experiment

Nutrients	Experiment	$S_0 \text{ (mg L}^{-1}\text{)}$	MR (mg L ⁻¹)	RE (%)	$k \text{ (d}^{-1}\text{)}$	$\lambda \text{ (d)}$	R ²	RMSE
N	C+	40 ± 1^a	14.1 ± 0.3^a	35.5 ± 0.7^a	0.4 ± 0.3	1 ± 2	0.945	1.265
	C-SE	10.67 ± 0.02^b	na	na	na	na	na	na
	SE	10.9 ± 0.5^b	5.66 ± 0.01^b	52.1 ± 0.1^b	0.8 ± 0.7	0.2 ± 1.9	0.986	0.227
	C-PE	29.78 ± 0.01^c	11.06 ± 0.02^c	37.13 ± 0.02^a	0.4 ± 0.3	1 ± 2	0.971	0.752
	PE	32.4 ± 0.5^c	28 ± 1^d	86 ± 4^c	0.8 ± 0.1	0.6 ± 0.2	0.999	0.410
P	C+	10.2 ± 0.1^a	5.5 ± 0.2^a	53 ± 2^a	0.6 ± 0.3	0.5 ± 1	0.972	0.347
	C-SE	2.4 ± 0.3^b	na	na	na	na	na	na
	SE	2.3 ± 0.1^b	2.02 ± 0.02^{bc}	86.5 ± 0.9^b	2.2 ± 0.2	0.3 ± 0.1	0.998	0.039
	C-PE	3.0 ± 0.3^b	1.8 ± 0.2^b	59 ± 3^c	0.9 ± 0.1	0.7 ± 0.3	0.997	0.039
	PE	3.0 ± 0.4^b	2.5 ± 0.1^c	80.0 ± 0.1^b	1.0 ± 0.2	0.2 ± 0.5	0.984	0.115

k: uptake rate; MR: mass removal; na: not applicable; RE: removal efficiency; RMSE; root mean squared error; R²: coefficient of determination; S_0 : initial nutrients concentration; λ : lag time. Values are presented as the mean $\pm$ standard deviation obtained from two independent experiments. Within the same column, and for the same element, average values sharing at least one common letter (a, b, c, and d) are not statistically different ($p > 0.05$).

The initial phosphorus concentrations were statistically higher ($p < 0.05$) in the modified OECD medium than in the filtered effluents and, therefore, the mass removal was also higher in these experimental conditions. Phosphorus concentration also remained constant throughout the C-SE assay, as represented in Figure 5 B, but a mass removal of $2.02 \pm 0.02 \text{ mg L}^{-1}$ was observed in the SE experiment due to bioassimilation by microalgae. In the C-PE experiment (Figure 5D), besides nitrogen removal, phosphorus concentration also decreased $1.8 \pm 0.2 \text{ mg L}^{-1}$. A possible explanation for this removal is the presence of polyphosphate accumulating organisms (PAO) in the PE effluent, bacteria that accumulate phosphorus in the form of polyphosphates within the cells (Tarayre et al., 2016). However, an even greater removal was observed in the PE assay, $2.5 \pm 0.1 \text{ mg L}^{-1}$, showing once again that the introduction of microalgae greatly improves nutrients removal. Furthermore, as opposed to the respective negative control, concentrations below the legislation limit were achieved within the cultivation time for the PE assay, after 6 days, as shown in Figure 5 D. No statistical difference was found between mass removal and removal efficiencies in the SE and PE assays ($p > 0.05$) since the initial phosphorus concentrations were identical. However, the SE cultures achieved concentrations below the legislation limit after only 2 days (Figure 5 B), sooner than the PE cultures. In accordance, a statistical difference ($p < 0.05$) was found between the uptakes rates obtained for these two experiments. The uptake rate in the SE assay was

higher, which can be related to the lower nutrients concentrations in this effluent. Initially, there was a lower nitrogen and phosphorus availability for the same biomass concentration and cell number in this experiment. This nutrients limitation might have induced microalgae to quickly assimilate phosphorus in the SE assay, resulting in a higher uptake rate. No statistical difference ($p > 0.05$) was found between the average lag times of each experiment.

Nitrogen removal efficiencies for SE (52%) and PE (86%) were lower than the ones obtained by Znad *et al.* (2018) for the filtered municipal secondary (83%) and primary effluents (100%), with initial nitrogen concentrations of 30.6 mg L⁻¹ and 11.8 mg L⁻¹, respectively. In the mentioned study, a mass removal of 30.6 mg L⁻¹ was obtained for the primary effluent, not very different from the mass removal in the PE assay from the present work, 27.9 mg L⁻¹. However, the mass removal was lower in the SE assay, 5.66 mg L⁻¹, compared to the value determined by Znad *et al.* (2018), 9.79 mg L⁻¹. A possible justification for these results could be the lower light intensity in the present study, resulting in lower photosynthetic efficiency and, consequently, lower growth and nutrient consumption. In terms of phosphorus removal, Znad *et al.* (2018) obtained removal efficiencies of 80% for the primary effluent, identical to the removal in the PE assay, and 100% for the secondary effluent, higher than the SE experiment (87%). In terms of mass removal, the values were much higher in the mentioned study. These differences might be due to the higher initial phosphorus concentrations of 6.6 mg L⁻¹ and 5.6 mg L⁻¹ for primary and secondary effluents, respectively, compared to this study: 2.5 mg L⁻¹ in the PE and 2.02 mg L⁻¹ in the SE.

Comparing these results with the growth behaviour in the SE and PE experiments, it is possible to observe that the stationary growth phase coincides with both nitrogen and phosphorus exhaustion. For instance, by day 3, phosphorus concentration in the SE assay reached very low concentrations, and microalgae could not assimilate this nutrient any further until the end of the experiment. However, they continued to consume nitrogen until a limiting concentration was reached on day 6, which is in accordance with the beginning of the stationary growth phase. Several studies have shown that nitrogen limitation in the culture medium can lead to lower biomass production and protein content, shifting the flux to other pathways such as lipid production (Yaakob *et al.*, 2021). Similar results were obtained in the PE assay, since by days 6 and 7, phosphorus and nitrogen concentrations, respectively, reached levels after which no removal was verified. Lastly, microalgae consume nutrients for activities other than cellular growth, such as the synthesis of nucleic acids and proteins (Liu & Chen, 2016). This removal is usually termed nutrients consumption for cellular maintenance, and it can explain some discrepancies observed between microalgal growth and nutrients removal.

4.2.2. Carbon

Due to its mixotrophic metabolism, *C. vulgaris* can perform mixotrophy and utilise different forms of carbon: inorganic carbon for photosynthesis and organic carbon for respiration (Siqueira et al., 2018). Figures 6 and 7 present the time-course evolution of COD and DIC/DOC/TDC, respectively, in each experiment. In the C+ assay, the initial DOC concentration was extremely low, $2 \pm 1 \text{ mg L}^{-1}$, as opposed to the DIC concentration, $52 \pm 4 \text{ mg L}^{-1}$ (Figure 7 E). These results are in accordance with the inorganic typology of the culture medium, which contains only inorganic carbon in the form of soluble carbonates from NaHCO_3 . Over the course of this experiment, an 86% decrease in DIC was verified. This removal is explained by the consumption of inorganic carbon by microalgae during photosynthesis, through carbon concentrating mechanisms that involve dehydration of HCO_3^- to CO_2 (Umetani et al., 2021). In contrast, the DOC concentration increased over time, reaching concentrations as high as $89 \pm 19 \text{ mg L}^{-1}$, most likely due to the excretion of metabolic products, such as soluble microbial products and EPSs (Otondo et al., 2018). These results are in accordance with the determined COD values that represent the amount of oxygen equivalents consumed in the chemical oxidation of organic matter by potassium dichromate. As shown in Figure 6 C, COD was low at the beginning of the experiment since there was nearly no organic matter, but increased in the last days of the experiment, due to the release of the mentioned metabolites.

In both effluents, the COD values were already below the EU legislation limit ($125 \text{ mg O}_2 \text{ L}^{-1}$). Compared to the raw effluents, the lower initial DOC concentrations in the filtered effluents show that the filtration process significantly removed organic matter. This pre-treatment step is very important for microalgal cultures since large organic solids cannot be assimilated directly by microalgae and can even hinder photosynthesis by increasing turbidity (Han et al., 2019). The addition of inoculum does not appear to have affected these parameters, since the initial DOC and DIC concentrations were not statistically different ($p > 0.05$) between the effluent-grown cultures and the respective negative controls. No significant differences ($p > 0.05$) were verified between the DOC/DIC values in the C-SE assay (Figure 7 A) on the first ($10 \pm 2 \text{ mg L}^{-1}/16 \pm 3 \text{ mg L}^{-1}$) and last days of the experiment ($10 \pm 1 \text{ mg L}^{-1}/15 \pm 1 \text{ mg L}^{-1}$). Even though there was a slight decrease in COD (Figure 6 A), the values were in a low concentration range and, therefore, these results do not appear indicative of organic matter removal. In the SE assay (Figure 7 B), DIC decreased from $17 \pm 2 \text{ mg L}^{-1}$ to $2.4 \pm 0.5 \text{ mg L}^{-1}$ (approximately 86%) due to the consumption of inorganic carbon for photosynthesis. On the other hand, DOC concentration increased from $9.8 \pm 0.8 \text{ mg L}^{-1}$ to $25 \pm 6 \text{ mg L}^{-1}$, as well as COD (Figure 6 A), most likely due to the excretion of metabolic products.

In parallel with nitrogen and phosphorus removal, DOC and DIC concentrations in the C-PE assay (Figure 7 C) decreased approximately 78% and 70% until day 6, to values within the same concentration range as in the C-SE assay, $14 \pm 1 \text{ mg L}^{-1}$ and $10.9 \pm 0.5 \text{ mg L}^{-1}$, respectively, probably due to the action of microorganisms in

the effluent. In the PE assay (Figure 7 D), DIC decreased approximately 94% until the end of the experiment, while DOC decreased 79% until day 6. The removal of organic matter was confirmed by a 20% decrease in COD for assay C-PE and 37% for assay PE, as shown in Figure 6 B. The slightly higher DOC and COD removal in the PE assay compared to the negative control suggests that *C. vulgaris* grew mixotrophically in these conditions. Therefore, the decrease in organic carbon was most likely a result of the combined action of microalgae and other microorganisms possibly present in the PE. However, after day 6, DOC started increasing due to the excretion of secondary metabolites during the stationary phase of microalgal growth.

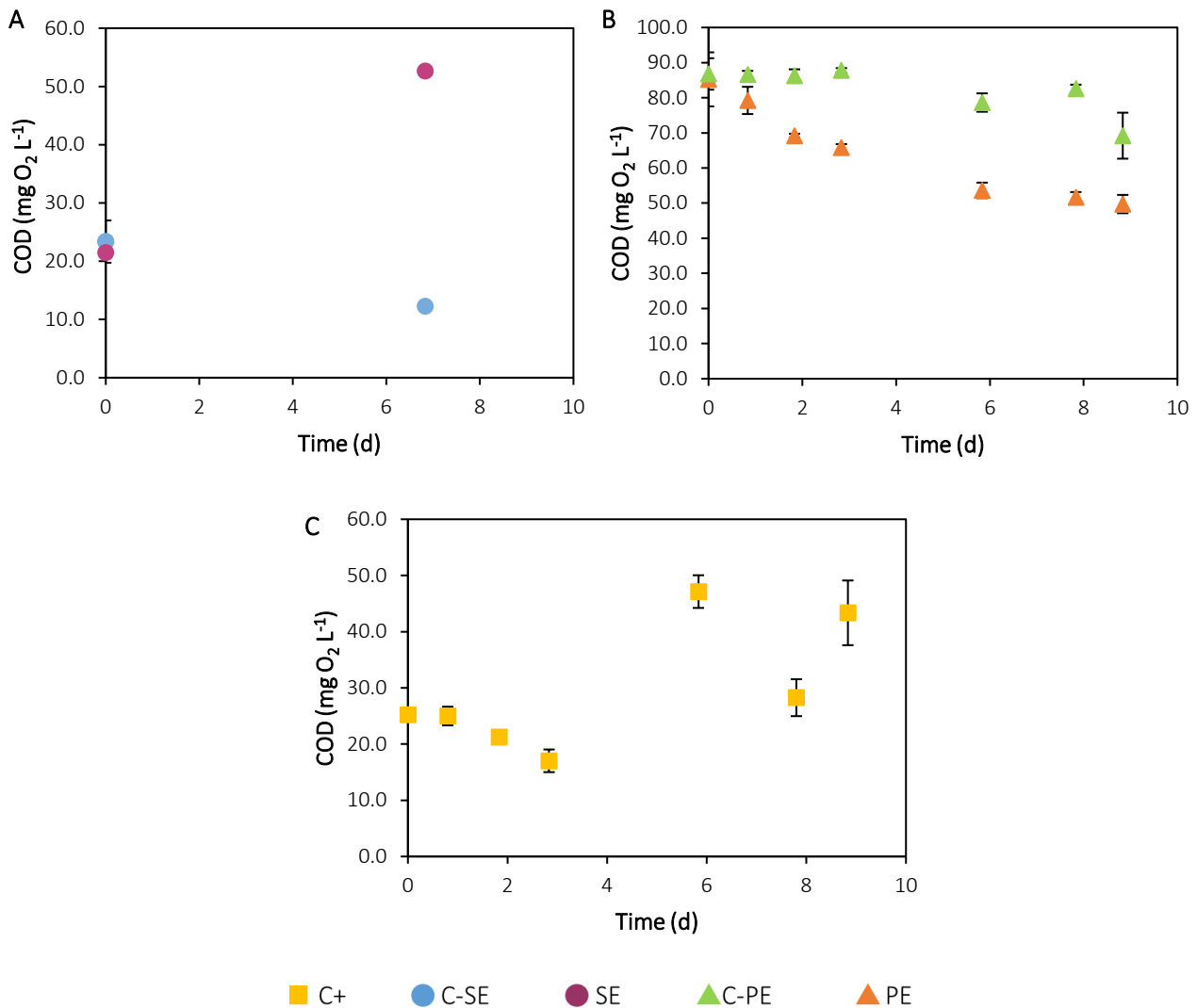


Figure 6 Time-course evolution of COD in assays C-SE/SE (A), C-PE/PE (B), and C+ (C). Error bars correspond to the standard deviation of the mean obtained from two independent experiments.

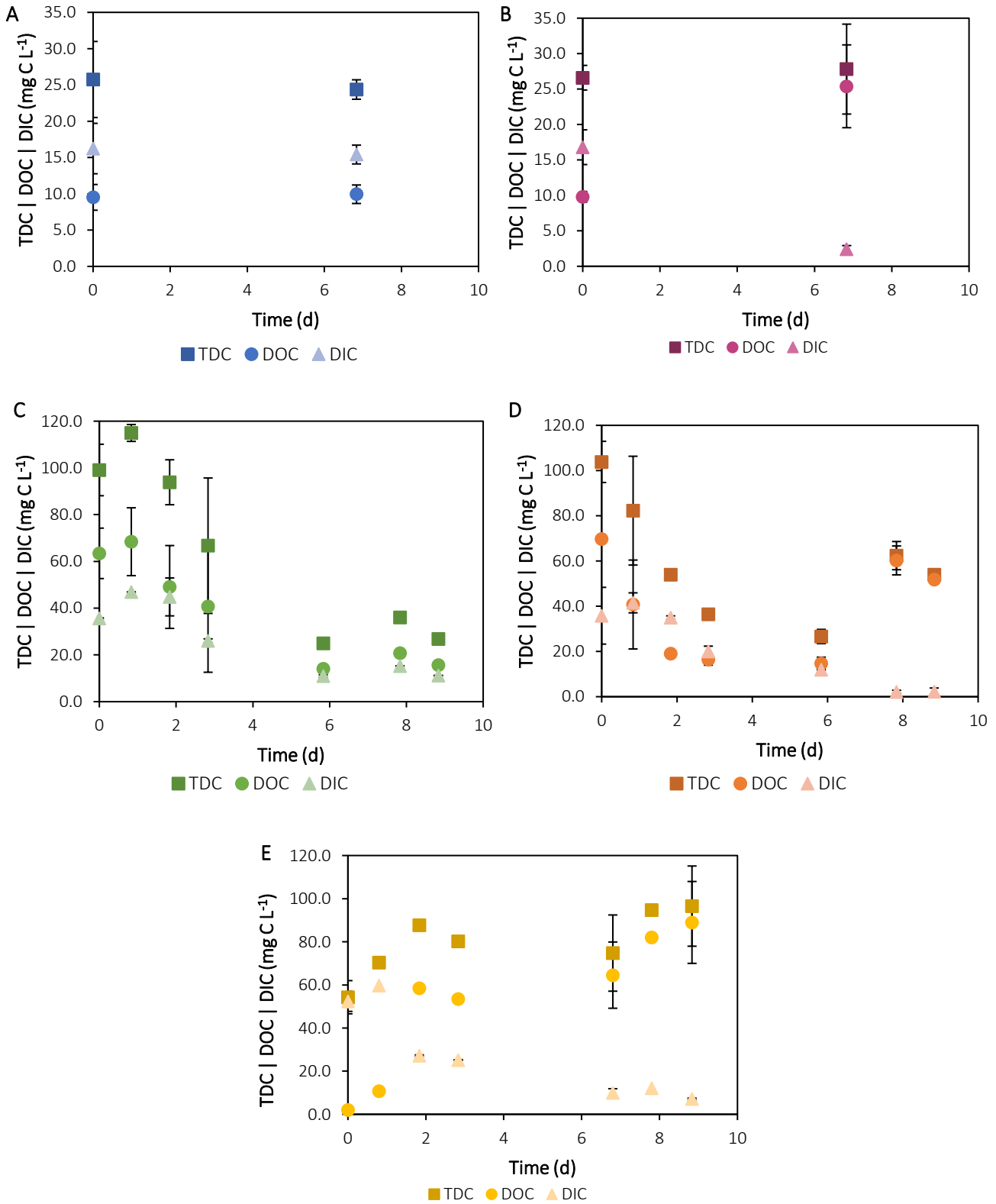


Figure 7 Time-course evolution of TDC, DOC and DIC in assays C-SE (A), SE (B), C-PE (C), PE (D) and C+ (E). Error bars correspond to the standard deviation of the mean obtained from two independent experiments.

4.3. Siloxanes removal

4.3.1. Air and water samples

VMSs were not detected in the collected air samples at the beginning and end of the C-SE and SE assays. D6 is not analysed in the GC-IMS-SILOX equipment, so it was not possible to assess its presence in these samples. However, D4 was detected in concentrations between 0.06 and 0.08 mg m⁻³ on the first day, not only for the C-PE and PE experiments, but also in the C+ assay. This could be evidence of external contamination of the cultures with D4. In the cells-free culture medium or water samples, all the studied cVMSs were detected but linear siloxanes L3 and L4 were not. Even though L5 was detected in both raw effluents at very low concentrations, no significant results were obtained for the variation of this IVMS in any of the experiments and, therefore, these results were not presented. Figure 8 shows the initial cVMSs concentrations in each experiment. Even though cautionary protocols were followed to avoid siloxane contamination, D3, D4, D5 and D6 were detected in the C+ assay and remained constant over the course of the experiment. These results can be derived from the presence of these compounds in the cultivation bottles and aeration tubes, or even from external contaminations during sample collection and pH adjustments. Nevertheless, the detected cVMSs concentrations in this assay were statistically lower ($p < 0.05$) when compared to the results obtained for the effluent-grown cultures. Overall, no statistical difference was found in cVMSs concentration between the effluent-grown cultures and the respective negative controls, which suggests that the inoculum did not contain these compounds.

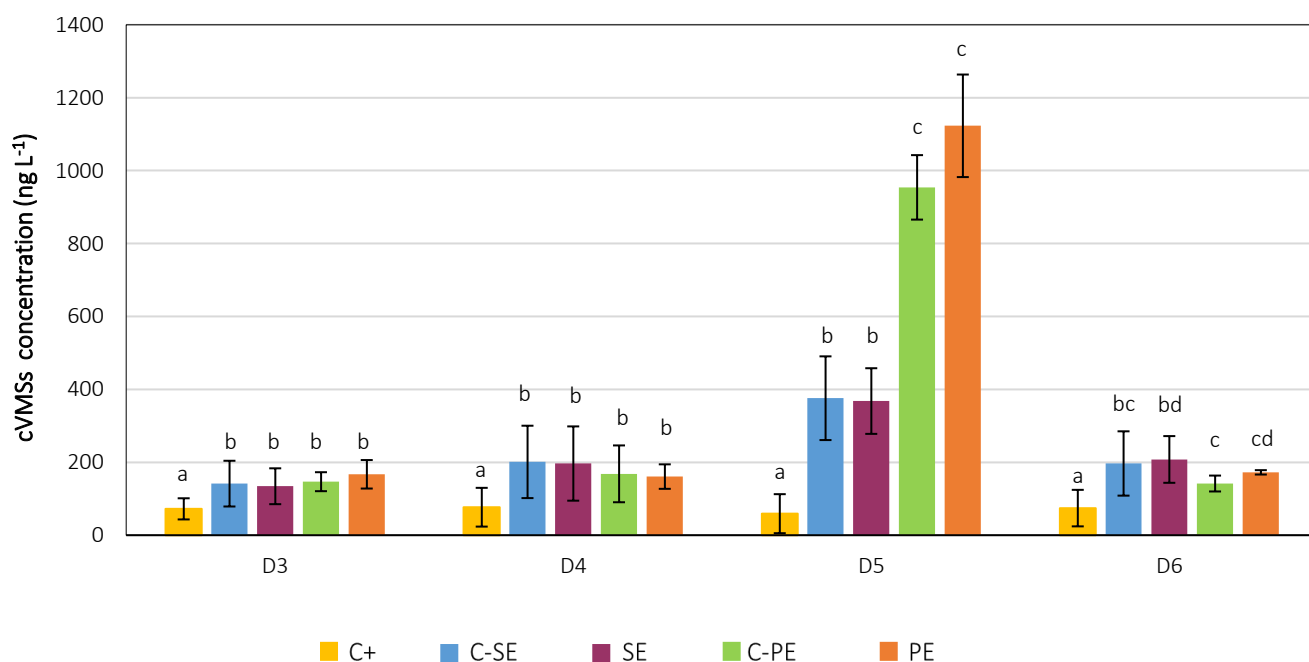


Figure 8 Initial D3, D4, D5 and D6 siloxane concentration in each experiment. Error bars correspond to the standard deviation of the mean obtained from two independent experiments. For each siloxane, average values sharing at least one common letter (a, b, c, and d) are not statistically different ($p > 0.05$).

D5 was the predominant siloxane in both PE and SE. This compound is one of the most problematic VMSs; its widespread daily use in PCPs leads to their substantial occurrence in wastewaters. The filtration process does not appear to have altered the concentration of this compound, since no statistical difference ($p > 0.05$) was found between the raw and the filtered effluents. Furthermore, D5 concentrations were statistically higher in the raw PE ($904 \pm 83 \text{ ng L}^{-1}$) compared to the raw SE ($455 \pm 141 \text{ ng L}^{-1}$). The lower concentration in the secondary-treated effluent can be explained by the significant aeration process occurring during secondary treatment in WWTPs, which can lead to the volatilisation of over 50% of the VMSs (Garcia et al., 2015). In both C-SE and SE assays, no removal was verified since D5 concentrations remained approximately constant over the course of the experiment, with an average of $376 \pm 115 \text{ ng L}^{-1}$ and $368 \pm 90 \text{ ng L}^{-1}$, respectively. However, in the C-PE and PE assays, a significant decrease was observed. Figure 9 shows the evolution of D5 with time in these assays, in which an identical removal of approximately 98% was seen for both experiments. As mentioned in Section 3.3, a negative control bottle without light exposure was tested. Comparing the results obtained for these two negative controls, identical values for all VMSs were verified, hence removal by photodegradation was ruled out. Therefore, the main removal pathway for D5 was most likely volatilisation. Another hypothesis for the removal of D5 in the negative control could be a combination of volatilisation with D5 degradation by bacteria present in the effluent, even if the latter was at lower extent. However, after one day of culturing, D5 concentration in the PE assay was significantly lower than in the negative control, which might indicate that the presence of microalgae potentiated this removal on the first days of the experiment. D5 is a lipophilic compound with high K_{ow} and K_{oc} and low water solubility, favouring its partition from the effluents to both air and microalgal biomass matrices. Therefore, even though aeration might be responsible for the removal of a great portion of the D5 load after the 9 days of culturing, the presence of microalgae might be useful in cases where it is necessary to reduce D5 levels in a shorter period.

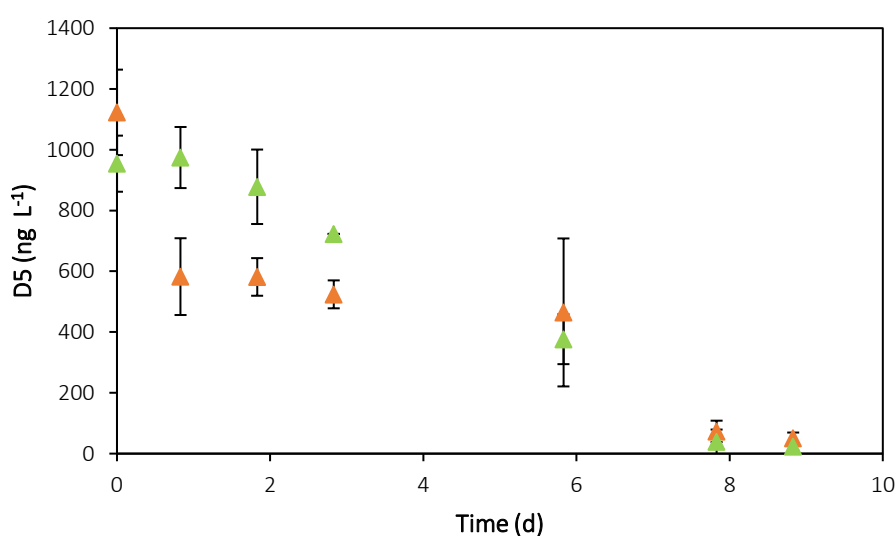


Figure 9 Time-course evolution of D5 concentration in the cells-free culture medium samples collected from C-PE and PE assays. Error bars correspond to the standard deviation of the mean obtained from two independent experiments.

D3 concentration was not statistically different ($p > 0.05$) between the raw SE ($127 \pm 35 \text{ ng L}^{-1}$) and PE ($184 \pm 88 \text{ ng L}^{-1}$). Furthermore, the filtration process does not appear to have influenced the concentration of this cVMS in the effluents as the values were not statistically different ($p > 0.05$) between the raw and the filtered effluents. Over the course of the C-SE and SE experiments, no significant variations were observed, and average D3 concentrations were $142 \pm 63 \text{ ng L}^{-1}$ and $134 \pm 49 \text{ ng L}^{-1}$, respectively. Time-course evolution of D3 concentration in the cells-free culture medium from assays C-PE and PE is presented in Figure V.1 A of Appendix V. It is possible to observe that the average D3 concentrations did not vary significantly between each day, as they remained between 124 and 238 ng L^{-1} . However, due to the high standard deviations associated with these mean concentrations, it is difficult to conclude on a tendency for the variation of D3 concentration over time. This high variability might be related to the fact that D3 has the highest vapour pressure and lowest boiling point of the studied VMSs and, therefore, it is a highly volatile siloxane.

No statistical difference was found ($p > 0.05$) between D4 and D6 concentrations in the raw SE ($246 \pm 16 \text{ ng D4 L}^{-1}$ and $233 \pm 39 \text{ ng D6 L}^{-1}$) and PE ($198 \pm 85 \text{ ng D4 L}^{-1}$ and $247 \pm 21 \text{ ng D6 L}^{-1}$). The filtration process does not appear to have affected the concentration of D4 in both effluents and D6 in the secondary effluent since there was no statistical difference between the detected values in the raw and filtered effluents. However, the initial concentrations of D6 in the C-PE and PE assays were statistically lower ($p < 0.05$) than the raw effluent. Since D6 is the cVMSs with the lowest water solubility, highest molecular weight, highest K_{ow} and K_{oc} values, this highly lipophilic compound was most likely attached to the organic matter in the PE. During the filtration process of this effluent, there was a significant removal of organic matter and, therefore, D6 might have been retained in the residue, which led to a reduced concentration in the filtered effluent. Identically to D3 and D5, no significant variations were verified in D4/D6 concentrations over the course of the C-SE and SE experiments, with average values of $201 \pm 99 \text{ ng D4 L}^{-1}/197 \pm 88 \text{ ng D6 L}^{-1}$ and $197 \pm 102 \text{ ng D4 L}^{-1}/208 \pm 64 \text{ ng D6 L}^{-1}$, respectively. These results might indicate that microalgae were not able to remove these siloxanes from the SE. Since variabilities were not detected in the negative control, it appears that volatilisation did not occur. However, cVMSs are volatile compounds thus it seems unlikely that these compounds did not volatilise at all over the course of the experiment, especially since air was bubbled continuously through the culture, which favours the partition from the liquid to the gaseous phase. Under these circumstances, it seems that the extraction and analysis methodologies used in this study were not sensible enough to possible variations in this low range of cVMS concentrations. Time-course evolution of D4 and D6 concentration in the cells-free culture medium from assays C-PE and PE is presented in Figure V.1 B and C of Appendix V, respectively. Even though the concentrations in the first and last two days of the experiments were similar, the concentrations for days 2, 3, 6 and 7, varied greatly throughout the experiments without an apparent pattern, even between duplicate samples. Even with all precautions, these compounds are highly volatile, and possible external contaminations

with D4 and D6 could have occurred during sample collection in these days, which could justify some of the higher values.

4.3.2. Biomass samples

D4, D5 and D6 were detected in the lyophilised biomass samples. During the QuEChERS extraction procedure, the sonication steps can contribute to cell rupture, as well as the development of a MgSO_4 solid agglomerate, which during the homogenisation steps can cause mechanical rupture. Therefore, the detected siloxanes in the biomass samples may have been removed from the wastewaters by bioadsorption and/or bioaccumulation mechanisms. Figure 10 shows the determined cVMSs concentration in terms of mass of siloxanes per mass of lyophilised samples. In the biomass from the C+ assay, only D4 was detected at a concentration of $0.8 \pm 0.8 \text{ ng g}_{\text{DW}}^{-1}$, much lower compared to the PE assay. The high standard deviation reflects the high variability between the two independent experiments, showing that D4 was present in these samples, most likely due to external contamination, which is in accordance with the results from the air samples.

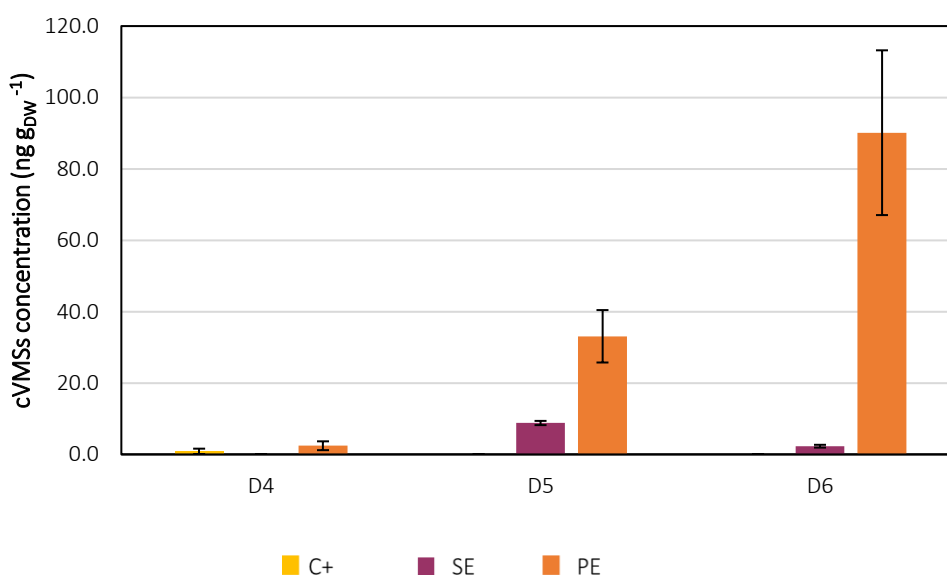


Figure 10 D4, D5 and D6 concentration in the lyophilised biomass samples collected from cultures C+, SE and PE.

In the SE assay, only D5 and D6 were detected, at concentrations $8.8 \pm 0.6 \text{ ng g}_{\text{DW}}^{-1}$ and $2.3 \pm 0.6 \text{ ng g}_{\text{DW}}^{-1}$, respectively. In the biomass samples from the PE assay, D4, D5 and D6 concentrations were $2 \pm 1 \text{ ng g}_{\text{DW}}^{-1}$, $33 \pm 7 \text{ ng g}_{\text{DW}}^{-1}$ and $90 \pm 23 \text{ ng g}_{\text{DW}}^{-1}$, respectively. The amount of D5 and D6 was significantly higher in the biomass samples from the PE compared to the SE. To understand to what extent cVMSs accumulated in the microalgal biomass, it is necessary to compare the mass of siloxanes in the biomass from the whole culture with the initial and final amounts of siloxanes in each experiment. Figure 11 shows the estimated mass of cVMSs in the biomass as well as in the cells-free culture media (water) at the first and last days of the SE and PE experiments. These values were obtained as described in Appendix IV. Figure V.2 of Appendix V presents the relative mass distribution of D3, D4, D5 and D6 in these assays. It is important to note that the discrepancies

between the initial and final mass of siloxanes in the aqueous phase are not necessarily related to a decrease in their concentrations, but to a decrease in volume and mass loss due to sample collection throughout the experiments.

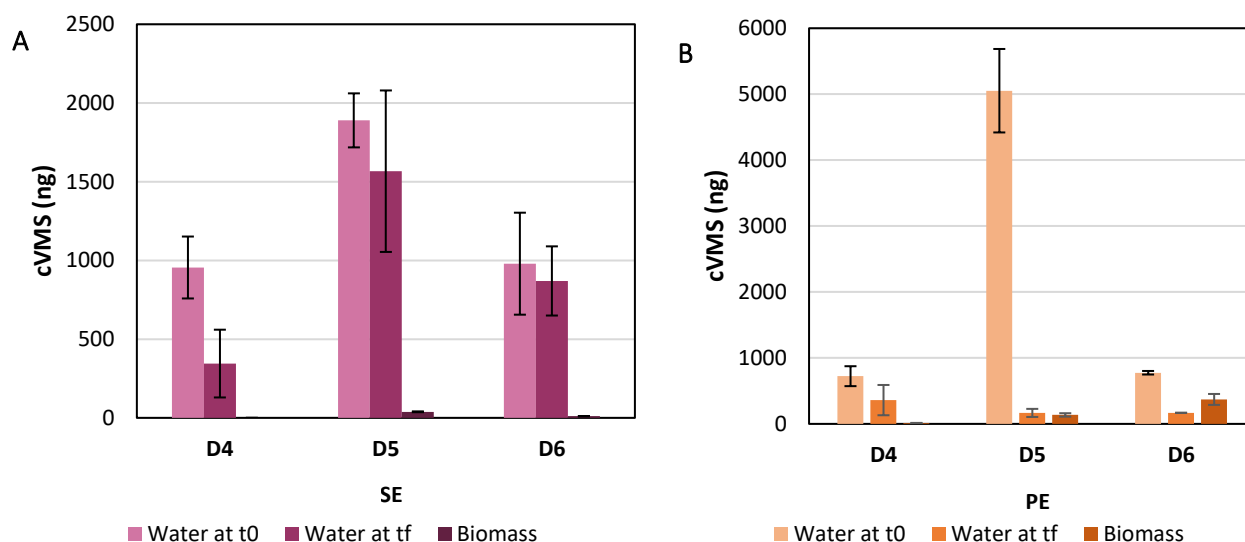


Figure 11 Estimated mass of siloxanes in biomass and water at the first and last days of the SE (A) and PE (B) assays.

D4 was not found in the biomass samples from the SE assay, and 36% remained in the cells-free culture medium. In the PE assay, 1% of the initial amount of D4 in the filtered effluent was in the biomass, while 50% remained in the aqueous phase on the last day of the experiment. For D5, on the last day of the SE experiment, approximately 2% of the initial amount of D5 was in the microalgal biomass, while 83% remained in the culture medium. For the PE, an identical percentage was obtained for D5 in biomass (3%), but only 3% remained in the aqueous phase. These results confirm that microalgae contributed to D5 removal but at a small extent, according to the time-course variation of D5 concentration in water samples from C-PE and PE assays. Lastly, in the SE experiment, 89% of the amount of D6 in the filtered effluent was in the culture medium, while 1% was in the microalgal biomass. In the PE assay, D6 was the VMS that accumulated to a greater extent in the microalgal biomass, since 48% of the initial mass of D6 was detected in the biomass samples while 22% remained in the aqueous phase. As mentioned before, D6 is the cVMS with the lowest water solubility, highest molecular weight, highest K_{ow} and K_{oc} values and, therefore, it is highly lipophilic. All these physicochemical properties contribute to higher adsorption of D6 onto the surface of microorganisms such as microalgae, compared to the remaining cVMSs. Overall, these results suggest that when *C. vulgaris* was cultured in the PE, there was an absorption or accumulation of siloxanes in biomass to a greater extent, compared to the culture of the same species in the same experimental conditions, but in the secondary effluent. Therefore, the composition of the PE appears to be more suitable for the study of siloxanes removal by microalgae.

5. Concluding remarks and future work

5.1. General conclusions

C. vulgaris grew successfully in both primary and secondary urban WWTP effluents, with specific growth rates identical in both experimental conditions: $0.036 \pm 0.002 \text{ d}^{-1}$ for the culture grown in the SE and $0.034 \pm 0.001 \text{ d}^{-1}$ for the PE. In terms of nutrients, the results showed that this microalga can efficiently remove nitrogen and phosphorus when cultured in these effluents, reaching values below the EU legislation limits within the cultivation time. In the SE assay, removal efficiencies of 52% for nitrogen and 87% for phosphorus were observed, and after 2 days of cultivation, nutrients levels were below the legislation limits. Removal of both nitrogen (37%) and phosphorus (59%) was verified in the C-PE assay, most likely due to the action of microorganisms present in the PE, such as nitrifying microorganisms, denitrifying aerobic bacteria and PAO. However, nutrients concentrations did not reach the legislation limits within the cultivation time. On the other hand, higher nitrogen (86%) and phosphorus (80%) removal efficiencies were verified in the PE assay, and after 6 days, these levels were below the legislation limits. These results show that there was a significant improvement in nutrients removal when *C. vulgaris* was present. Regarding carbon removal, after 6 days of cultivation, there was a significant decrease in both DIC and DOC in this assay. These results show that microalgal cultures are an economical and environment-friendly option for both secondary and tertiary wastewater treatments.

D5 was the predominant VMS in both PE ($904 \pm 83 \text{ ng L}^{-1}$) and SE ($455 \pm 141 \text{ ng L}^{-1}$), with lower concentrations detected in the SE probably due to the aeration process during the secondary treatment step in WWTPs. A decrease in D5 concentration of approximately 98% was verified in the culture medium over the course of the PE assay, most likely due to volatilisation. Three out of the seven analysed VMSs were detected in the biomass samples (D4, D5 and D6), but D6 was the one that accumulated to a greater extent, since 48% of the initial mass of D6 was detected in the biomass samples while 22% remained in the aqueous phase. Moreover, the results suggest that when *C. vulgaris* was cultured in the PE, absorption or accumulation of siloxanes in biomass might have occurred to a greater extent, compared to the culture of the same species in the same experimental conditions, but in the SE. Therefore, the composition of the PE appears to be more suitable for the study of siloxanes removal by microalgae.

In conclusion, the proposed objectives for the present work were accomplished successfully and the results evidence the remarkable potential of *C. vulgaris* in bioremediation of wastewaters, indicating a possible ability to remove specific VMSs from effluents, at least to a certain extent. However, different experimental conditions must be tested for an increased understanding of this subject.

5.2. Limitations and future work

The main limitation during the development of this work was the scale of the cultures. Since the remaining culture volume on the last day of the experiments was necessary to recover microalgal biomass, sample collection over the course of the experiment had to be managed not to compromise the amount of recovered biomass. Culturing *C. vulgaris* at a larger scale would allow: (i) siloxane analysis in the culture medium in triplicates and higher volume in each sample to amplify the signal and decrease variability; (ii) higher quantity of biomass recovered at the end of the experiment, which could be useful to perform recovery tests on the QUECHERS method. Additionally, in future experiments, optimised methods for VMSs extraction from microalgal biomass can be developed and validated, since the QuEChERS extraction procedure used in the present work was an adaptation of the method developed for liquid sludge.

Another limitation was the low concentrations of siloxanes in the collected effluents. Higher concentrations would most likely decrease the variability in the analytical response and allow the observation of a siloxane concentration tendency over the course of the experiments. Furthermore, a higher siloxanes concentration promotes an increased contact between the microalgal cells and these molecules, which might stimulate adsorption, accumulation and possible biodegradation. The VMSs concentration in wastewaters may vary considerably with the seasons and meteorological conditions. For instance, during the winter, wastewaters are more diluted and, therefore, siloxane concentration tends to be lower. Since these wastewaters were collected in the winter/beginning of spring, further testing with wastewaters from different seasons is recommended. Furthermore, testing with industrial wastewaters could solve this limitation, since they could contain higher levels of these compounds. Another option could be spiking the real wastewaters with individual VMSs and evaluate their removal separately.

If the removal of siloxanes by microalgae is possible in these conditions, the type of mechanisms involved in this removal (bioadsorption, bioaccumulation or biodegradation) could be evaluated. To distinguish between bioaccumulation and bioadsorption, an initial extraction procedure without cellular rupture must be performed, followed by another one that promotes this process. To evaluate if microalgae can degrade siloxanes, the presence of biological degradation intermediates, such as DMSD and elemental biomass composition in terms of Si could be assessed. Lastly, if the removal of siloxanes from water by microalgae is proven in these new experimental conditions, the treatment of an effluent with siloxanes using activated sludge, microalgae cultures and consortia between bacteria and microalgae could be further studied. Moreover, the removal of these compounds not only focusing on wastewaters but also on biogas using microalgal cultures could be explored.

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Appendix I: Composition of the modified OECD culture medium

Table I.1 shows the composition of the modified OECD microalgae culture medium. This culture medium is prepared by adding 10 mL of solution A, 1 mL of solution B, 1 mL of solution C and 10 mL of solution D (prepared separately) and complete the volume to 1 L using distilled water.

Table I.1 Composition of the modified OECD microalgae culture medium

Solution	Reagents	Concentration in the media (mg L ⁻¹)	Concentration in the solution (g L ⁻¹)
A	NaNO ₃	250	25
	MgCl ₂ ·6H ₂ O	12	1.2
	CaCl ₂ ·2H ₂ O	18	1.8
	MgSO ₄ ·7H ₂ O	15	1.5
	KH ₂ PO ₄	45	4.5
B	FeCl ₃ ·6H ₂ O	0.08	0.08
	Na ₂ EDTA·2H ₂ O	0.1	0.1
C	H ₃ BO ₃	0.185	0.185
	MnCl ₂ ·4H ₂ O	0.415	0.415
	ZnCl ₂	3×10 ⁻³	3×10 ⁻³
	CoCl ₂ ·6H ₂ O	1.5×10 ⁻³	1.5×10 ⁻³
	CuCl ₂ ·2H ₂ O	0.01×10 ⁻³	0.01×10 ⁻³
	Na ₂ MoO ₄ ·2H ₂ O	7×10 ⁻³	7×10 ⁻³
D	NaHCO ₃	500	50

Appendix II: Calibration curves

Table II.1 presents the data from the calibration curves used for the development of this work. The limits of detection (LOD) and quantification (LOQ) correspondent to these calibration curves, were determined according to Equations II.1 and II.2, in which s_b corresponds to the standard deviation of the intercept and a represents the slope of the calibration curve.

Table II.1 Calibration curves data for several parameters evaluated in this study

x	y	$a \pm s_a$	$b \pm s_b$	R^2	LOD	LOQ	n_s
Biomass concentration ($g_{DW} L^{-1}$)	OD _{680 nm}	2.50 ± 0.09	-2.40 ± 0.11	0.996	0.13	0.45	5
NO ₃ ⁻ ($mg L^{-1}$)	Abs _{410 nm}	$1.19 \times 10^{-2} \pm 1.18 \times 10^{-4}$	$4.59 \times 10^{-2} \pm 3.31 \times 10^{-3}$	1.00	0.83	2.78	7
KH ₂ PO ₄ ($mg L^{-1}$)	Abs _{820 nm}	$6.19 \times 10^{-2} \pm 7.92 \times 10^{-4}$	$-5.70 \times 10^{-3} \pm 2.88 \times 10^{-2}$	0.999	1.4	4.6	6
TP ($mg L^{-1}$)	Abs _{880 nm}	$6.06 \times 10^{-1} \pm 5.94 \times 10^{-3}$	$3.90 \times 10^{-3} \pm 1.67 \times 10^{-3}$	0.997	0.008	0.028	7
Colour (HU)	Abs _{400 nm}	$9.28 \times 10^{-4} \pm 7.66 \times 10^{-6}$	$-2.23 \times 10^{-3} \pm 1.31 \times 10^{-3}$	0.999	4.2	14	13
COD- low range ($mg O_2 L^{-1}$)	Abs _{410 nm}	$2.76 \times 10^{-3} \pm 6.79 \times 10^{-5}$	$9.67 \times 10^{-3} \pm 3.25 \times 10^{-3}$	0.997	3.5	12	7
COD- high range ($mg O_2 L^{-1}$)	Abs _{410 nm}	$4.23 \times 10^{-4} \pm 6.05 \times 10^{-6}$	$2.33 \times 10^{-3} \pm 3.25 \times 10^{-3}$	0.999	23	77	7

a: slope of the calibration curve; Abs: absorbance; b: intercept; COD: chemical oxygen demand; DW: dry weight; HU: Hazen units; KH₂PO₄: monopotassium phosphate; LOD: limit of detection; LOQ: limit of quantification; NO₃⁻: nitrate; n_s : number of standards; OD: optical density; R^2 : coefficient of determination; s_a : standard deviation of the slope; s_b : standard deviation of the intercept; TP: total phosphorus.

$$LOD = \frac{3 \times s_b}{a} \quad (II.1)$$

$$LOQ = \frac{10 \times s_b}{a} \quad (II.2)$$

Appendix III: Microalgal growth monitoring and sample frequency

Time-course evolution of temperature and pH in each experiment is represented in Figure III.1, and the determined OD values are presented in Figure III.2. Table III.1 presents a summary of the sample collection days in each experiment. Table III.2 shows the colour and turbidity measurements of the cells-free culture media at the beginning and end of each experiment.

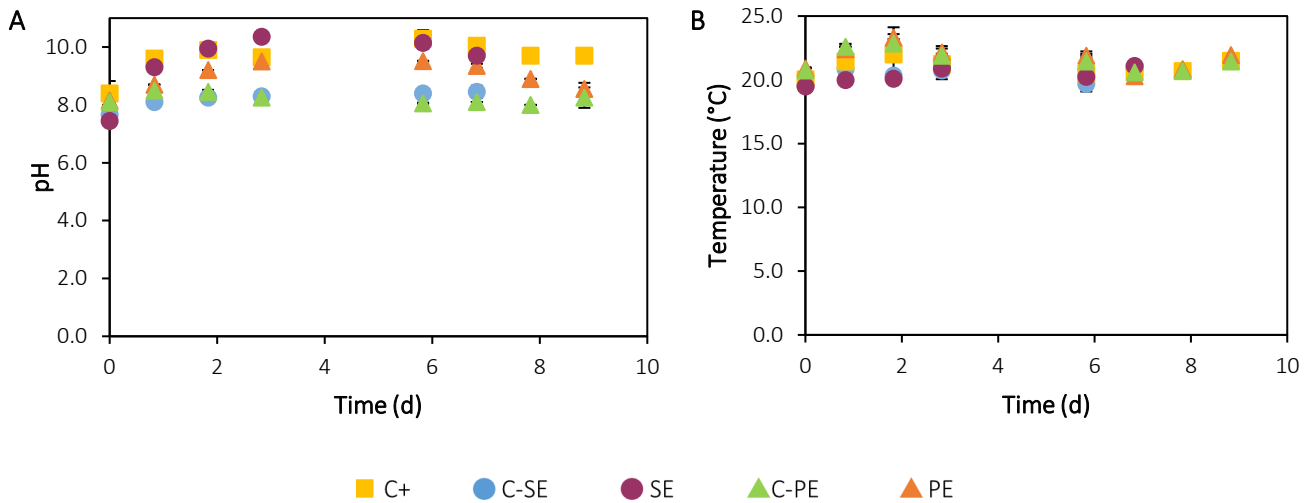


Figure III.1 Time-course evolution of pH (A) and temperature (B) in each experiment. Error bars correspond to the standard deviation of the mean obtained from two independent experiments.

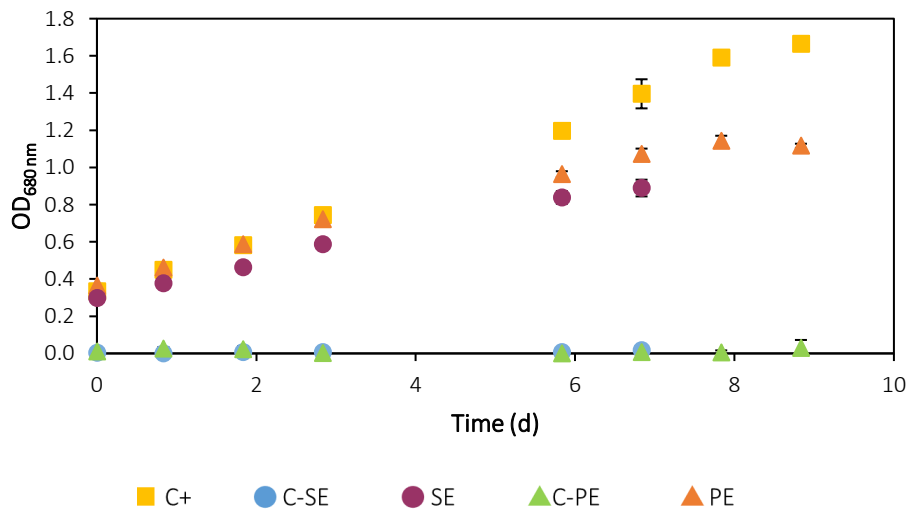


Figure III.2 Time-course evolution of OD_{680 nm} in each experiment. Error bars correspond to the standard deviation of the mean obtained from two independent experiments.

Table III.1 Summary of collection days for the analysed samples

Parameter	Culture sample days			Sample volume per day (mL)
	C+	C-SE / SE	C-PE / PE	
Optical density	0, 1, 2, 3, 6, 7, 8, 9	0, 1, 2, 3, 6, 7	0, 1, 2, 3, 6, 7, 8, 9	5
Biomass dry weight	0, 2, 3, 6, 8, 9	0, 2, 3, 6, 7	0, 2, 3, 6, 8, 9	25
TN, DOC, TDC, DIC and COD	0, 1, 2, 3, 6, 8, 9	0, 7	0, 1, 2, 3, 6, 8, 9	50
PO ₄ -P and NO ₃ -N	0, 1, 2, 3, 6, 7, 8, 9	0, 1, 2, 3, 6, 7	0, 1, 2, 3, 6, 7, 8, 9	15
VMSs in water	0, 1, 2, 3, 6, 7, 8, 9	0, 1, 2, 3, 6, 7	0, 1, 2, 3, 6, 7, 8, 9	70
VMSs in air	0, 9	0, 7	0, 9	1 000
Colour and turbidity	0, 9	0, 7	0, 9	25

COD: chemical oxygen demand; DIC: dissolved inorganic carbon; DOC: dissolved organic carbon; DW: dry weight; NO₃-N: nitrate-nitrogen; PO₄-P: phosphate-phosphorus; TN: total nitrogen; TDC: total dissolved carbon; VMSs: volatile methylsiloxanes. For the negative controls, samples for the determination of biomass concentration were not collected.

Table III.2 Colour and turbidity measurements of the cells-free culture media at the beginning and end of each experiment

Parameter	Experiment	Time	
		t ₀	t _f
Colour (HU)	C+	6.4 ± 0.5*	10.5 ± 0.8*
	C-SE	29 ± 7 ^a	20 ± 3 ^a
	SE	30 ± 1 ^a	55 ± 7 ^b
	C-PE	76 ± 8 ^a	47 ± 10 ^b
	PE	65 ± 6 ^a	68 ± 3 ^a
Turbidity (NTU)	C+	0.9 ± 0.3 ^a	1.8 ± 0.9 ^a
	C-SE	0.8 ± 0.2 ^a	0.70 ± 0.09 ^a
	SE	1.2 ± 0.3 ^a	2.4 ± 0.9 ^a
	C-PE	5.5 ± 0.4 ^a	2.8 ± 0.4 ^b
	PE	4.8 ± 0.8 ^a	3.3 ± 0.5 ^b

HU: Hazen units; NTU: Nephelometric turbidity unit; t₀: time correspondent to the beginning of the experiment (d); t_f: time correspondent to the end of the experiment (d); *values below the method's limit of quantification. Values are presented as the mean ± standard deviation obtained from two independent experiments; different letters (a, b) within the same row represent statistically different values ($p < 0.05$).

Appendix IV: Siloxanes quantification

Tables IV.1 and IV.2 represent the calibration curve data for each VMS in the water and biomass samples, respectively.

Table IV.1 VMSs calibration curve data correspondent to the water samples

x ($\mu\text{g L}^{-1}$)	y	$a \pm s_a$ ($\text{L } \mu\text{g}^{-1}$)	R^2	LOD ($\mu\text{g L}^{-1}$)	LOQ ($\mu\text{g L}^{-1}$)	n_s
C_{L5}	A_{L5}/A_{IS}	$4.61 \times 10^{-3} \pm 4.47 \times 10^{-5}$	0.999	0.0053	0.018	9
C_{D3}	A_{D3}/A_{IS}	$5.43 \times 10^{-3} \pm 6.06 \times 10^{-5}$	0.999	0.14	0.45	11
C_{D4}	A_{D4}/A_{IS}	$5.30 \times 10^{-3} \pm 8.58 \times 10^{-5}$	0.997	0.18	0.59	11
C_{D5}	A_{D5}/A_{IS}	$2.94 \times 10^{-3} \pm 1.12 \times 10^{-5}$	1.00	0.0059	0.020	9
C_{D6}	A_{D6}/A_{IS}	$3.48 \times 10^{-3} \pm 5.43 \times 10^{-5}$	0.998	0.11	0.36	11

C_{VMS} : volatile methylsiloxanes concentration; L5: dodecamethylpentasiloxane; D3: hexamethylcyclotrisiloxane; D4: octamethylcyclotetrasiloxane; D5: decamethylcyclopentasiloxane; D6: dodecamethylcyclohexasiloxane; a: slope of the calibration curve; s_a : standard deviation of the slope; R^2 : coefficient of determination; LOD: limit of detection; LOQ: limit of quantification; n_s : number of standards.

Table IV.2 VMSs calibration curve data correspondent to the biomass samples

x ($\mu\text{g L}^{-1}$)	y	$a \pm s_a$ ($\text{L } \mu\text{g}^{-1}$)	R^2	LOD ($\mu\text{g L}^{-1}$)	LOQ ($\mu\text{g L}^{-1}$)	n_s
C_{L5}	A_{L5}/A_{IS}	$2.10 \times 10^{-3} \pm 1.33 \times 10^{-5}$	0.999	0.0084	0.028	9
C_{D3}	A_{D3}/A_{IS}	$2.97 \times 10^{-3} \pm 4.76 \times 10^{-5}$	0.998	0.19	0.64	9
C_{D4}	A_{D4}/A_{IS}	$2.56 \times 10^{-3} \pm 3.65 \times 10^{-5}$	0.998	0.25	0.85	9
C_{D5}	A_{D5}/A_{IS}	$1.49 \times 10^{-3} \pm 2.45 \times 10^{-5}$	0.998	0.0042	0.014	9
C_{D6}	A_{D6}/A_{IS}	$1.73 \times 10^{-3} \pm 2.56 \times 10^{-5}$	0.998	0.13	0.43	9

a: slope of the calibration curve; C_{VMS} : volatile methylsiloxanes concentration; D3: hexamethylcyclotrisiloxane; D4: octamethylcyclotetrasiloxane; D5: decamethylcyclopentasiloxane; D6: dodecamethylcyclohexasiloxane; LOD: limit of detection; LOQ: limit of quantification; L5: dodecamethylpentasiloxane; n_s : number of standards; R^2 : coefficient of determination; s_a : standard deviation of the slope.

The LOD and LOQ for each calibration curve correspondent to a specific VMS were determined according to Equations IV.1 and IV.2, in which C_{LSTD} corresponds to the lowest concentration standard in ng L^{-1} and SNR_{LSTD} represents the signal-to-noise ratio obtained by the GC-MS for that standard.

$$\text{LOD} = \frac{3 \times C_{LSTD}}{SNR_{LSTD}} \quad (\text{IV.1})$$

$$\text{LOQ} = \frac{10 \times C_{LSTD}}{SNR_{LSTD}} \quad (\text{IV.2})$$

The concentration of a certain VMS in a water sample, C_w (ng L^{-1}), was determined according to Equation IV.3, in which: (i) $\left(\frac{A_{VMS}}{A_{IS}}\right)_{ws}$ is the ratio between the peak area for the VMS and the peak area for the IS for the water sample; (ii) $\left(\frac{A_{VMS}}{A_{IS}}\right)_{LB}$ is the ratio between the peak area for the VMS and the peak area for the IS

correspondent to the LB; (iii) a is the slope of the respective calibration curve in $\mu\text{g L}^{-1}$; (iv) V_{cs} is the concentrated sample volume (0.5 mL) and (v) V_s is the sample volume (30 mL).

$$C_w = \frac{\left(\frac{A_{VMS}}{A_{IS}}\right)_{ws} - \left(\frac{A_{VMS}}{A_{IS}}\right)_{LB}}{a} \times \frac{V_{cs}}{V_s} \times 10^3 \quad (\text{IV.3})$$

The estimated mass of a certain VMS in the cells-free culture medium at the beginning of each experiment, M_{w0} (ng), was calculated as represented in Equation IV.4, in which C_{w0} is the VMS concentration of the samples collected at this time (ng L^{-1}) and V_0 corresponds to the initial culture volume (L). Equation IV.5 represents the determination of the estimated VMS mass at the end of each experiment, M_{wf} (ng), where C_{wf} corresponds to the VMS concentration of the samples collected at this time (ng L^{-1}) and $V_{samples}$ represents the total volume of the samples collected over the course of each experiment (L).

$$M_{w0} = C_{w0} \times V_{t0} \quad (\text{IV.4})$$

$$M_{wf} = C_{wf} \times (V_0 - V_{samples}) \quad (\text{IV.5})$$

The VMS concentration in lyophilised biomass samples, C_b (ng L^{-1}), was determined according to Equation IV.6, in which $\left(\frac{A_{VMS}}{A_{IS}}\right)_{bs}$ is the ratio between the peak area for the VMS and the peak area for the IS corresponding to the biomass sample. The mass of VMS per mass of lyophilised biomass samples, $C_{s/b}$ ($\text{ng g}_{\text{DW}}^{-1}$), was calculated as represented in Equation IV.7, where V_e is the extract volume (1 mL) and M_b is the amount of lyophilised biomass (g_{DW}) used in the extraction procedure for that sample. The estimated amount of VMS in biomass at the end of the experiment, in each culture, M_b (ng), was calculated according to Equation IV.8, where X_f corresponds to the final biomass concentration in $\text{g}_{\text{DW}} \text{L}^{-1}$.

$$C_b = \frac{\left(\frac{A_{VMS}}{A_{IS}}\right)_s - \left(\frac{A_{VMS}}{A_{IS}}\right)_{LB}}{a} \times 10^3 \quad (\text{IV.6})$$

$$C_{s/b} = \frac{C_b \times V_e}{M_{lb}} \quad (\text{IV.7})$$

$$M_b = C_{s/b} \times (V_0 - V_{samples}) \times X_f \quad (\text{IV.8})$$

Appendix V: Time-course evolution of siloxanes concentration

Figure V.1 presents the time-course evolution of D3, D4 and D6 concentrations for the C-PE and PE experiments. Figure V.2 shows the relative mass distribution of D3, D4, D5 and D6 in these assays.

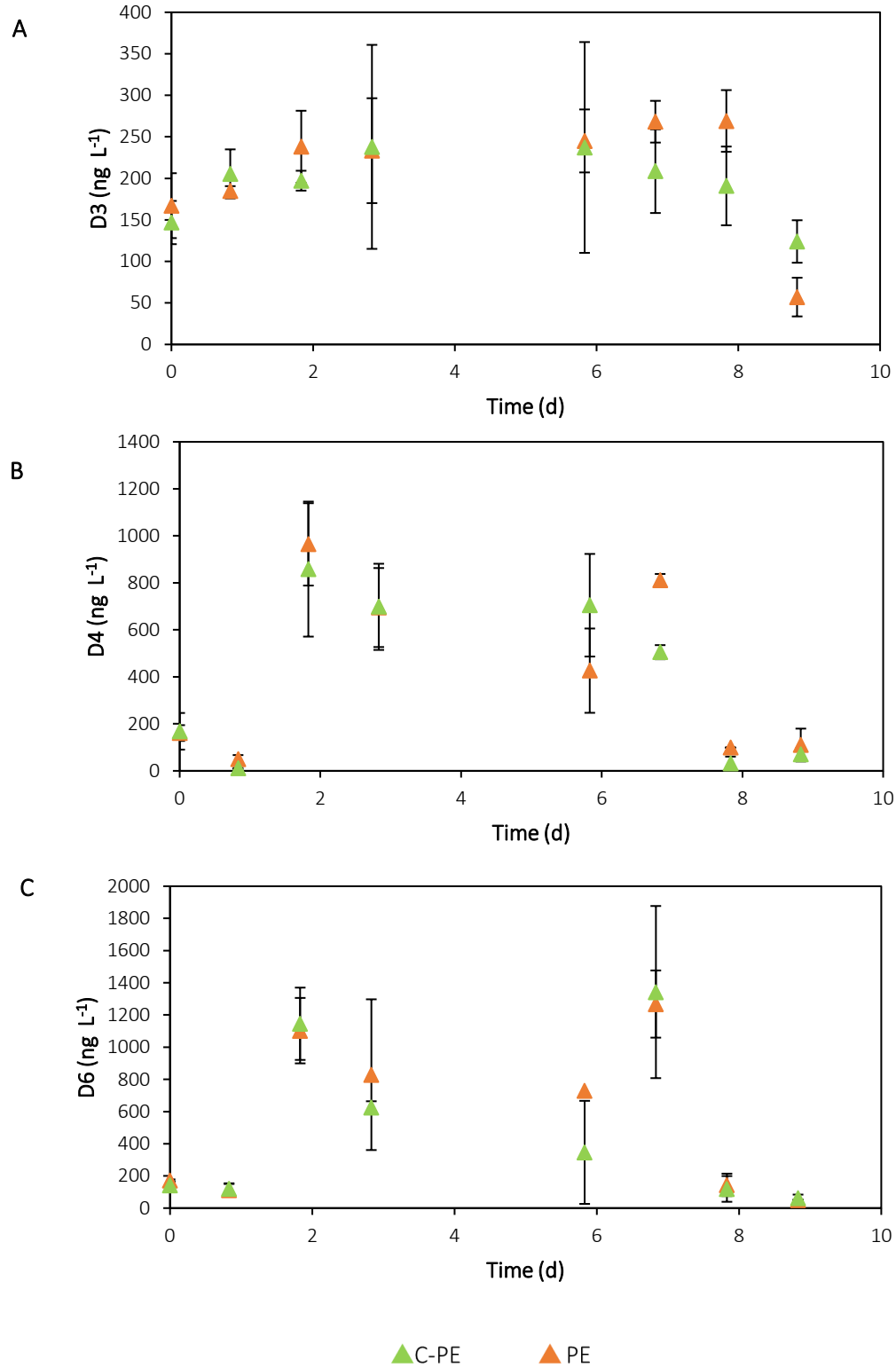
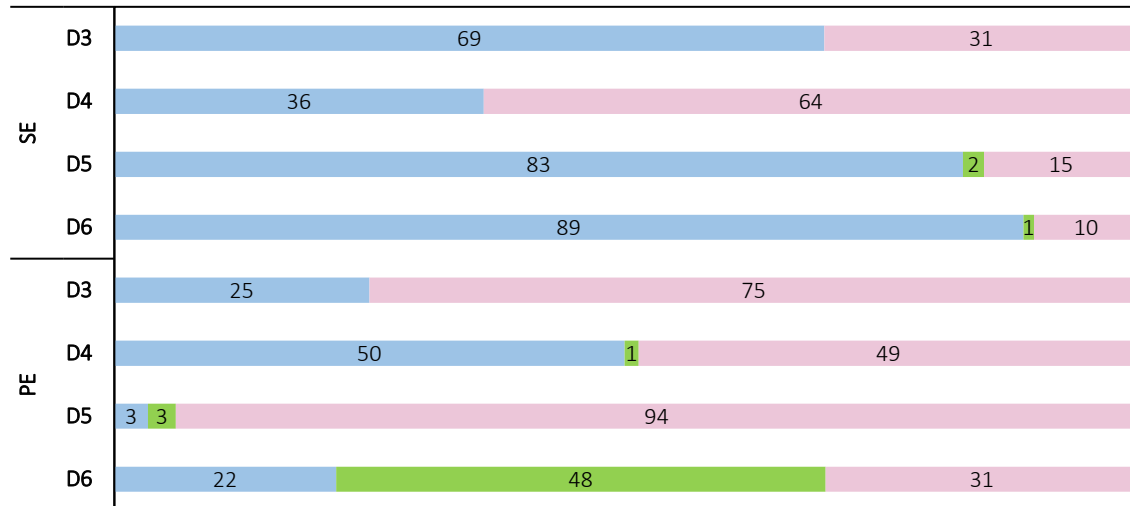


Figure V.1 Time-course evolution of D3 (A), D4 (B) and D6 (C) concentrations for the C-PE and PE experiments.



■ % of VMS in the culture medium at t_f ■ % of VMS in the microalgal biomass at t_f ■ % of VMS removed from t_0 to t_f

Figure V.2 Mass distribution (%) of D3, D4, D5 and D6 in the SE and PE assays.