Universidade do Minho
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Insights into the role of biocides in the performance of activated-sludge process focusing on the filamentous populations

April 2022

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Doctoral Thesis
Doctorate in Chemical and Biological Engineering

Work developed under supervision of:
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April 2022

ACKNOWLEDGEMENTS

No final desta intensa etapa científica, não posso deixar de me lembrar de todas as pessoas que de alguma forma contribuíram para este doutoramento, ajudando-me a torná-lo um pouco mais fácil e acima de tudo, inesquecível. Em primeiro lugar, gostaria de agradecer às minhas orientadoras, Doutora Ana Nicolau e Professora Doutora Maria Olívia Pereira, por me terem desafiado a iniciar este doutoramento, pela confiança que sempre depositaram em mim, incentivo e carinho, e, acima de tudo, por terem partilhado comigo o fascínio desta área.

Ao Professor Doutor Manuel Mota, agradeço os ensinamentos e a ajuda nunca negada e, que sem dúvida, enriqueceram o meu trabalho. À Professora Doutora Madalena Alves, agradeço a disponibilidade e simpatia com que me recebeu no Grupo BRIDGE e no Laboratório de Biotecnologia Ambiental.

Agradeço a todos os colegas do BRIDGE e do LMAmb, a amizade e o companheirismo. Agradeço em especial à Marta, ao Gabriel, ao Jorge, à Rute e ao Rui. Obrigada pelos momentos de alegria, pelos conselhos, pela ajuda, e, acima de tudo, pelo bom ambiente. Assim, é bom trabalhar em equipa!

À Nicole e à Cláudia, agradeço a amizade, o estarem sempre lá para me ajudar. Vocês foram, e ainda são, dois anjos da guarda na minha vida. Agradeço também às amigas, Méninha, Paulinha, Susy e Thalita, por me ouvirem, por me aconselharem, por me fazerem rir, por estarem sempre do meu lado.

Não me posso esquecer de agradecer ao "fórum": Isa, Filipa e Lili. Uma amizade que começou numa mesa enquanto escrevíamos a tese de mestrado e que se mantém nos dias de hoje. Obrigada por tornarem a minha vida mais feliz e preenchida; mesmo com a distância, vocês estão sempre no meu coração.

Termino, por agradecer à minha Família, a quem estou profundamente agradecida pelo amor incondicional e orgulho, apoio constante, paciência e sem os quais eu não conseguiria ter chegado onde cheguei. E por fim, ao Miguel e Gonçalo, a quem dedico esta tese, por serem o meu porto de abrigo e a minha força nesta longa caminhada. *Obrigada por tudo!*

The work presented in this thesis was performed in the Centre of Biological Engineering (CEB), Minho University. Financial support was provided by the Portuguese Foundation for Science and Technology (FCT) and European Social Fund (POPH-QREN) through a research grant (SFRH/BD/80054/2011), by national funds through FCT under the scope of the strategic funding of UIDB/04469/2020 unit and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte; and by the FEDER funds through the project PTDC/AAG-TEC/3331/2014, financed under the Operational Competitiveness Programme (COMPETE), and by national funds through FCT. Also, the author gratefully acknowledges AGERE-E.M.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho

Compreensão sobre o papel dos biocidas no desempenho do processo de lamas ativadas com particular atenção nas bactérias filamentosas

RESUMO

O impacto ambiental relacionado com o uso de vários produtos químicos antimicrobianos (biocidas) na prática geral e em ambientes domésticos e industriais está na ordem do dia. A explosão de produtos antimicrobianos para lavagem de mãos e limpeza de superfícies é um bom exemplo da disseminação do uso de biocidas nos últimos anos. Estes têm uma contribuição significativa nos impactos das atividades humanas no meio ambiente e na saúde, principalmente relacionados com a seleção de estirpes resistentes a antibióticos.

O processo de lamas ativadas é o tipo de tratamento biológico mais utilizado para remover poluentes de águas residuais em todo o mundo devido às suas vantagens económicas. Diferentes tipos de microrganismos (bactérias, fungos e protozoários) estão envolvidos no processo de depuração das águas residuais. As bactérias correspondem a cerca de 95 % do total da comunidade microbiana presente nas lamas ativadas, sendo as principais responsáveis pela degradação de substâncias orgânicas das águas residuais. As bactérias filamentosas são componentes normais da biomassa das lamas ativadas; no entanto, o seu crescimento excessivo pode causar problemas na sedimentação das lamas (*bulking* filamentoso) e na formação de espumas (*foaming* filamentoso) reduzindo a eficiência das estações de tratamento de águas residuais (ETAR). Nesta tese de doutoramento, foi explorada uma nova abordagem para a potencial influência de biocidas sobre o desempenho global de processos de lamas ativadas, abordando-se o uso de biocidas como potenciais compostos de controlo das populações de bactérias filamentosas esclarecendo, simultaneamente, o impacto desses produtos químicos nas comunidades biológicas de uma ETAR.

Inicialmente, foi realizado um estudo de 16 ETAR Portuguesas de lamas ativadas, de forma a melhorar o conhecimento dos parâmetros ambientais que determinam a composição e a prevalência da comunidade filamentosa no tanque de arejamento. Um total de 22 morfotipos filamentosos foram identificados. O Tipo 1851 foi o morfotipo mais frequentemente dominante, seguido por *Microthrix parvicella* e os Tipos 0092 e 0041/0675. Estes também foram os morfotipos dominantes durante as ocorrências de *bulking* filamentoso. Foram obtidas correlações significativas entre a abundância de bactérias filamentosas e os parâmetros ambientais, mas a análise estatística multivariada apenas confirmou a correlação negativa entre o Tipo 0092 e o Índice Volumétrico de Lamas (IVL), enfatizando a associação deste organismo filamentoso com o *bulking*.

Numa segunda fase, foi avaliado o efeito *in-vitro* do triclosano [0.25 a 200 µg/mL], do brometo de cetiltrimetilamónio (CTAB) [1 a 40 µg/mL] e do glutaraldeído [40 a 1000 µg/mL] em duas bactérias filamentosas, *Nocardia amarae* (Gram positiva) e *Sphaerotilus natans* (Gram negativa). Os resultados mostraram um efeito "dose-dependente" na viabilidade bacteriana e diferentes mecanismos de ação. Além disso, diferenças na suscetibilidade à exposição de cada biocida sugeriram um efeito "estirpe-dependente". Todos os biocidas causaram fragmentação dos filamentos em células dispersas, perdendo-se também, por isso, a capacidade de produzir flocos. Após a remoção do biocida, as células permaneceram dispersas, e mesmo as bactérias viáveis não foram capazes de produzir novos filamentos em 48 horas. Entre os biocidas testados, o CTAB apresentou o maior efeito biocida, seguido do triclosano e glutaraldeído para *N. amarae*, mas no caso de *S. natans*, a ordem entre os dois primeiros inverteu-se, apresentando-se o triclosano como o mais tóxico.

Finalmente, foram conduzidos ensaios à escala laboratorial, de forma a avaliar os efeitos biocidas, utilizando amostras de lamas ativadas reais (simulando uma ETAR real), a fim de compreender as mudanças na comunidade microbiana num cenário mais complexo. Os resultados permitiram avaliar o efeito dos biocidas tanto na comunidade microbiana como nos parâmetros de desempenho das unidades de lamas ativadas estudadas. O efeito biocida foi percetível desde o início do ensaio no caso do triclosano (na concentração de 2 mg/g MLSS) e do CTAB e do glutaraldeído (a partir da concentração de 10 mg/g MLSS). As duas bactérias filamentosas dominantes mostraram reações diferentes aos biocidas: a Gram negativa Tipo 0092 demonstrou ser claramente mais resistente do que a Gram positiva Tipo 0041/0675. Conclui-se que a adição de biocidas originou fragmentação de bactérias filamentosas em células dispersas, perda de estrutura dos flocos (menor densidade).

Em termos globais, foi demonstrado que é possível aproveitar a presença de biocidas no desempenho global dos processos de lamas ativadas, particularmente no controlo do *bulking* filamentoso, desde que monitorizados os parâmetros de avaliação do processo, bem como o desempenho geral e estudos iniciais sejam realizados levando em consideração as bactérias filamentosas mais problemáticas.

Palavras chave: bactérias filamentosas; biocidas; controlo do *bulking* filamentoso; desempenho da ETAR; lamas ativadas

Insights into the role of biocides in the performance of activated-sludge process focusing on the filamentous populations

ABSTRACT

The environmental impact related to the use of antimicrobial chemicals (biocides), in general practice and in domestic and industrial settings, is in the order of the day. The explosion of antimicrobial hand-wash and cleaning products in recent years is a paradigmatic example of the increasing use of biocides. It is well known that their use has a significant contribution to the environmental and health impacts, mainly related with the selection of antibiotic-resistant strains.

The activated-sludge process is the most widely used type of biological treatment to remove pollutants worldwide, mainly due to its economic advantages. Different types of microorganisms (bacteria, fungi and protozoa) are involved in the wastewater processing and cleaning. Bacteria sum up around 95 % of the total microbial community of the activated-sludge, being responsible for most of the degradation of organic substances in the wastewater. Filamentous bacteria are normal components of activated-sludge biomass but their excessive growth leads to potential problems mainly on the sludge settling (filamentous bulking) or in the formation of scums (filamentous foaming), thus reducing the efficiency of the wastewater treatment plants (WWTP). In this thesis, a new approach to the potential influence of biocides on the overall performance of activated-sludge processes was explored, addressing the use of biocides as potential control compounds of the filamentous bacteria populations, but also aiming at clarifying the impact of these chemicals in the biological communities of WWTP.

At first, a study of 16 Portuguese activated-sludge WWTP was carried out to improve the understanding of the environmental parameters determining the composition and prevalence of the filamentous community in the aeration tank. A total of 22 filamentous morphotypes were identified. Type 1851 was the most frequently dominant morphotype, followed by *Microthrix parvicella* and Types 0092 and 0041/0675. These were also the dominant morphotypes during bulking occurrences. Significant correlations were obtained between the abundance of filamentous bacteria and environmental parameters, but multivariate statistical analysis only confirmed the negative correlation between Type 0092 and sludge volume index (SVI), emphasizing the association of this particular filamentous organism with bulking.

In a second phase, the *in-vitro* effect of triclosan [0.25 to 200 μg/mL], cetyltrimethyl ammonium bromide (CTAB) [1 to 40 μg/mL] and glutaraldehyde [40 to 1000 μg/mL] on two filamentous bacteria, *Nocardia amarae* (Gram positive) and *Sphaerotilus natans* (Gram negative), was assessed. The results showed a dose-dependent effect on bacterial viability and different action mechanisms. Moreover, differences in susceptibility to exposure of each biocide suggested a strain-dependent effect. All biocides led to morphological modification of the filaments leading to their fragmentation to single cells. Therefore, the ability to enhance floc formation through the filament skeleton was also lost. After biocide removal, the morphology remained as single cells, even in viable bacteria, which were not able to produce new filaments in the next 48 h. Among the biocides tested, CTAB was found to present the highest biocidal effect, followed by triclosan and glutaraldehyde for *N. amarae*, whereas for *S. natans*, the ranking of toxicity was triclosan, CTAB and glutaraldehyde.

Finally, bench-scale assays were conducted to evaluate biocide global effects using samples of real activated-sludge (mimicking a real WWTP). Simultaneously the performance of the plants was assessed. The results allowed to infer the effect of biocides both in the microbial community and in the performance of activated-sludge units. The biocidal effect was perceptible since the very beginning of the assay for triclosan at the lowest concentration (2 mg/g MLSS), but for CTAB and glutaraldehyde, the effect was only noticeable at the concentration of 10 mg/g MLSS. The two dominant filamentous bacteria, Type 0092 and 0041/0675, showed different susceptibility to the biocides and, in this case, the Gram negative Type 0092 was clearly more resistant then the Gram positive Type 0041/0675. It was also possible to infer that the biocides addition gave rise to fragmentation of filamentous bacteria into single cells and loss of floc structure (lower density).

In global terms, it has been shown that it is possible to take advantage of the presence of these biocides in the overall performance of activated-sludge processes, particularly in the filamentous bulking control, as long as the other components and overall performance are monitored and initial studies are accomplished taking in consideration the problematic filamentous bacteria.

Keywords: activated-sludge; biocides; filamentous bacteria; filamentous bulking control; WWTP performance

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AF	Antifadent
Ag	Silver
AGERE-EM	Empresa de Águas, Efluentes e Resíduos de Braga, EM
ATP	Adenosine triphosphate
BOD	Biological oxygen demand
BOD_5	Five-day biochemical oxygen demand
BP	Band-pass filter
$Ca_{10}(PO_4)_6(OH)_2$	Calcium hydroxyapatite
$CaCO_3$	Calcium carbonate
Cd	Cadmium
Cl	Chlorine
CO_2	Carbon dioxide
COD	Chemical oxygen demand
CTAB	Cetyltrimethyl ammonium bromide
Cu	Copper
DAPI	4,6-diamidino-2-phenylindole
DBNPA	Dibromonitrilopropionamide
DGGE	Denaturant gradient gel electrophoresis
DGH	Dodecylguanidine hydrochloride
DMSO	Dimethylsulfoxide
DO	Dissolved oxygen
EBPR	Enhanced biological phosphorus removal
ECC	European Community Commission
EM	Emission spectra
ENR	Enoyl-acyl carrier protein reductase
EPS	Extracellular polymeric substances
ETAR	Estação de tratamento de águas residuais
EX	*Excitation* spectra
F/M	Food to microorganism ratio
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate

h	Hour(s)
H_2S	Hydrogen sulphide
HCL	Hydrochloric acid
Hg	Mercury
HOBr	Hypobromous acid
HOCl	Hypochlorous acid
HRT	Hydraulic retention time
IC_{50}	Half maximal inhibitory concentration
LC_{50}	Lethal concentration for half of the test organisms
MAR	Microautoradiography
MBT	Methylene bis-thiocyanate
MCRT	Mean cell residence time
min	Minute(s)
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
N	Nitrogen
N_2	dinitrogen gas
N_2O	Nitrous oxide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_4^+	Ammonium
NH_4–N	Ammonium nitrogen
Ni	Nickel
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NO_3–N	Nitrate nitrogen
O_2	Oxygen
OD	Optical density
OUR	Oxygen uptake rate
P	Phosphorus
Pb	Lead
PI	Propidium iodide

QACs	Quaternary ammonium compounds
rpm	Rotation per minute
SBI	Sludge biotic index
SBR	Sequencing batch reactors
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SOUR	Specific oxygen uptake rate
SRT	Solids residence time
SVI	Sludge volume index
SYTO-BC	SYTO dyes bacterial count
T	Temperature
TN	Total nitrogen
TP	Total phosphorus
TRITC	Tetramethylrhodamine
TSS	Total suspended solids
v/v	Percent volume/volume
vs	*Versus*
WWTP	Wastewater treatment plant(s)
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide

LIST OF SYMBOLS

DO_2	Dissolved oxygen concentration at the finish of the test	mg/L
DO_1	Dissolved oxygen concentration at the start of the test	mg/L
T_d	Doubling time	h
k	Growth rate constant	1/h
y	Bacterial growth at time x	
µM	Micromolar concentration	µmol/L
X	MLVSS concentration	mg/L
$SOUR_c$	Specific respiration rate of the control	mg/g MLVSS.h
$SOUR_t$	Specific respiration rate of the tested biocide concentration	mg/g MLVSS.h
Δt	Test duration time	h
x	Time	h
y_0	Value y at time 0	
λ	Wavelength of absorbance	nm

CHAPTER 1

Introduction

This chapter presents, in a brief introduction, the research motivation and the main objectives pursued in this dissertation. A summary of the following chapters is also provided.

1.1 Research motivation

To meet the wide–ranging increasing needs for water, urgent measures must be taken to ensure the quality of this resource. It is well known that water sources withstand frequent dramatic changes both in microbial and chemical quality as a result of the variety of activities on the watershed. These transformations are dramatically affecting ecosystems (López-Doval *et al.* 2017; Goonetilleke & Lampard 2019). In underdeveloped countries, and unhappily in some of the developed countries, discharges of untreated or inappropriately treated municipal wastewaters to the receiving waters, such as streams, rivers, lakes, ponds etc., have been identified as the main events responsible for these changes (Sibewu *et al.* 2008; Naidoo & Olaniran 2014). Additionally, new and emerging pollutants present a new global water quality challenge with potentially-serious threats to human health and ecosystems (Benson *et al.* 2017; Östman *et al.* 2018). So, it is imperative that wastewater treatment plants (WWTP) treat adequately the residual waters before discharging them into the receptive water bodies.

The activated-sludge process is the biological treatment option more frequently used worldwide to treat municipal and industrial wastewaters. Though generally considered a cost-effective and efficient technology, since its development in 1914, operation problems and deterioration of treatment efficiency due to bulking sludge (a condition defined by solids with poor settling characteristic), among others, have been cited as common problems in activated-sludge processes around the word (Pal *et al.* 2014; Scholes *et al.* 2016; Masłoń *et al.* 2019). The identification of the problems of WWTP at the level of their microbial communities is recognized as an essential component of the monitoring of these systems. Protozoa, members of the microbial assemblages of activated sludge, play an important role in the amount of freely-suspended and loosely-attached bacterial cells in the mixed-liquor, taking part in the reduction of suspended solids and turbidity in the final effluent. As a result, protozoa significantly contribute to a better performance of the system (Amanatidou *et al.* 2016). At the same time, bacteria have a fundamental role in the biological removal of organic carbon, ammonium and phosphate in the aeration tank. However, the overgrowth of filamentous bacteria, which are considered as normal components of the activated-sludge microbial community, is generally identified as the main origin of two types of harmful phenomena: sludge bulking and foaming, which, among other inconveniences, deteriorate sludge settleability and the quality of the final effluent, endangering the performance of WWTP (Cao & Lou 2016; Fan *et al.* 2018).

In activated-sludge systems, the performance of the process largely depends on the balance between filamentous and floc-forming bacteria populations (Wágner *et al.* 2015; Gurjar & Tyagi 2017). When this balance is disturbed by variations of physical-chemical and operational conditions, filamentous bacteria can proliferate causing the referred problems. Although numerous studies have attempted to develop methods to control the filamentous organisms commonly identified in WWTP (Eikelboom 2000; Jenkins *et al.* 2004; Pal *et al.* 2014), no definitive solution exists to control filamentous bacteria.

The raising of emergent biocides and their increasing use in both house-cleaning products and medical disinfectants has increased the attention of researchers because of the potential effects of their dissemination to human health and to natural environments (Hahn *et al.* 2010; Mamais *et al.* 2011; Lupo & Berendonk 2012; Stalder *et al.* 2012; Petrie *et al.* 2015; Batt *et al.* 2017;), but, on the other hand, has also attracted the attention of wastewater treatment technicians and researchers for the potential of their use in the control of filamentous overgrowth (Seka *et al.* 2001; Xie *et al.* 2007; Bodík *et al.* 2008; Mamais *et al.* 2011; Guo *et al.* 2012; Xu *et al.* 2014; Amat *et al.* 2015).

Therefore, the potential influence of biocides on the overall performance of activated-sludge processes deserves to be investigated. The assessment of in-use biocides as potential control compounds of the filamentous bacteria populations must clarify the impact of these chemicals in the biological communities of WWTP, including side-effects on the various populations inhabiting the activated-sludge plants.

1.2 Objectives

The mail goal of this study was to evaluate the effects of the introduction of biocides, such as phenolic compounds, quaternary ammonium compounds and aldehyde-based biocides, in the overall performance of activated-sludge, and particularly in the filamentous bulking control. The specific objectives were:

1. to assess the prevalence of filamentous bacteria in 16 nutrient-removal Portuguese WWTP located in the North of Portugal (region Minho) and to identify the conventional environmental factors related with their prevalence in aerated tanks; these WWTP are considered urban WWTP: they receive domestic and industrial sewage in different amounts, and even in the case of significant amounts of industrial residual water, this is not quantitatively or qualitatively defined and varies along time;

2. to *in-vitro* assess the effect of quaternary ammonium compounds, aldehyde-based biocides and phenolic compounds on specific filamentous bacterial populations;

3. to evaluate the action of the referred biocides on the microbial community and on the performance of activated-sludge at lab-scale.

1.3 Thesis outline

The present dissertation is divided into six chapters. Three of them describe experimental laboratory work (CHAPTER 3 to CHAPTER 5), each one starting with a brief introduction, followed by experimental methods and procedures, results and discussion and the conclusions obtained for each of the chapters. Supplementary material also appears at the end of some chapters, presenting the supportive content that is useful for understanding the chapter, although not essential to the argument of the results. The remaining two chapters provide information on the pertaining literature and on the most relevant conclusions (CHAPTER 2 and CHAPTER 6, respectively).

A brief introduction to the subject of this dissertation presenting the context, motivation and principal objectives are presented in **CHAPTER 1** (current chapter).

CHAPTER 2 summarizes the state-of-the-art of activated-sludge systems and the associated problems, such as filamentous bulking and foaming in WWTP, including the problematic of their identification. Moreover, a description of the actual scenario of biocide uses, the influence of biocides on the performance of activated-sludge process and a brief overview on how their presence might be an effective control strategy, are also introduced and described in this chapter.

In **CHAPTER 3**, a study on the prevalence of the filamentous bacteria in 16 Portuguese urban nutrient-removal WWTP is presented. The establishment of correlations with environmental parameters contribute to identify the factors potentially determining the composition of the filamentous bacterial populations in the aeration tank.

CHAPTER 4 presents the main results regarding the *in-vitro* studies of the effect of three selected biocides on specific filamentous bacteria, *Nocardia amarae* and *Sphaerotilus natans*.

The potential toxic effect of emergent biocides on the microbial community and on the performance of activated-sludge was evaluated in a bench-scale system, using mixed liquor and real sewage. A

respirometric assay was conducted as a preliminary evaluation of the biocide impact on the microbial community, using mixed liquor and real sewage from three different WWTP. Results of these studies are presented and discussed in **CHAPTER 5**.

Finally, **CHAPTER 6** contains the most significant conclusions derived from the conducted studies and the suggestions for future research in this field. Also, in this chapter, the main scientific outputs of the thesis are outlined.

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CHAPTER 2

Literature review

Most WWTP withstand the well-known phenomenon of filamentous bulking, the failure of the sedimentation and of the sludge thickening processes due to overgrowth of filamentous with respect to floc-forming microorganisms. Moreover, an excessive growth of certain filamentous bacteria can also lead to the formation of scums (or foam), a phenomenon known as filamentous foaming. These two events are the most common causes of malfunctioning of activated-sludge WWTP and have attracted an increasing attention of the scientific community in searching for solutions. The purpose of this chapter is to provide a review of the main subjects addressed along the thesis. In the first part of this chapter, the water problematic and the environmental problems raised by the contamination of wastewater with pollutants is examined. In the second and third parts of this chapter, special attention is given to the activated-sludge systems, the filamentous overgrowth associated problems, such as filamentous bulking and foaming, and the factors contributing to these phenomena. The fourth part of this chapter focus the use of biocides, namely triclosan, cetyltrimethyl ammonium bromide (CTAB) and glutaraldehyde, their effects in the community of activated-sludge systems and their potential use as control measures of filamentous overgrowth.

2.1 Wastewater treatment

2.1.1 Water problematic

Despite the considerable investment in water supply and sanitation, diseases associated with poor water quality and wastewater treatment still are a public health concern, especially in the developing world. Even in developed countries, industry, trade, agriculture, health units and household activities constitute significant threats water quality, by generating large volumes of wastewater (Abel 1996). This wastewater contains high quantities of pollutants, namely organic matter, nutrients (Nitrogen (N) and Phosphorus (P)), hydrocarbons and dissolved salts, pathogenic microorganisms, heavy metals, detergents and others, depending on its source. Since the natural ecosystems have no capacity to eliminate, in time, the pollutants in the amounts in which they are discarded, treatment before their discharge is necessary. The need for pollution control required the development and implementation of specific legislation on wastewater treatment and discharge, in order to minimize the subsequent impact in the environment, so that the consequences of the final effluent discharge are the minimum possible (Abel 1996; Martins 2004).

On another perspective, the present work addresses the goals of the Agenda 2030 of United Nations, namely Goal 6 (Ensure availability and sustainable management of water and sanitation for all); and, in this, namely 6.3 (by 2030, improve water quality by reducing pollution, eliminating dumping and minimizing release of hazardous chemicals and materials, halving the proportion of untreated wastewater and substantially increasing recycling and safe reuse globally) and 6.6 (by 2020, protect and restore water-related ecosystems, including mountains, forests, wetlands, rivers, aquifers and lakes): even being considered of relatively easy use and management, wastewater treatment by activated-sludge suffers from recurrent performance slumps mainly caused by filamentous overgrowth leading to sludge washout and the respective contamination of water and soil and related ecosystems. As the treated effluents of activated-sludge wastewater treatment plants are universally used to irrigation, namely lawns and golf courses, this being a crescent tendency, the malfunctioning of the wastewater treatment plants restrains the recycling and safe reuse of water.

2.1.2 Water pollution

There are numerous water pollutants whose effects are of actual or potential concern. This may be related with problems in fresh water reservoirs, rivers, estuaries and sea, such as contamination, eutrophication, decreased capacity for sediment accumulation and biodiversity reduction (Abel 1996). One of the most

severe forms of pollution occurs through the so-called "diffuse sources" in which the pollutant does not enter the water from a single point: it is case of the cumulative effect of various small introductions of contaminants gathered in large areas, for instance, through leaching of landfills, mines, agriculture, and other industries, contamination by acidification, plant nutrients and pesticides. Although the periodic monitoring of reservoirs, and others, such as rivers, etc, is usually carried on by water agencies, the analysis of other non-regulated substances and emergent pollutants is often not included or not extensive. For example, the European Water Framework Directive (ECC 2000) indicates the parameters to be supervised in lakes and reservoirs and rivers, etc (primarily nutrients and other basic indicators of trophic state and water quality), but the analysis of many chemical substances is not mandatory (López-Doval *et al.* 2017). Nevertheless, the effect of most of the pollutants are known for a long time (Table 2.1).

Table 2.1: Main pollutants in urban wastewater (adapted from Metcalf & Eddy, 2013, Carr *et al.*, 2016 and Spataro *et al.*, 2019)

Component	Specific component	Environmental effect
Biodegradable organic matter	Low Dissolved Oxygen (DO)	Death of fish, and other aquatic species, by asphyxiation, odours
Other organic matter	Detergents, pesticides, oils and fats dyes, solvents, phenols, cyanides	Toxins, aesthetic inconveniences, accumulate in the food chain
Nutrients	N and P	Eutrophication, decreased oxygen concentration, toxic effect
Metals	Mercury (Hg), Lead (Pb), Cadmium (Cd) Copper (Cu), Nickel (Ni)	Toxic effect, bioaccumulation
Other inorganic materials	Acids and bases	Corrosion, toxic effect
Thermal effects	High temperatures	Alteration of the living conditions of the fauna, flora and microorganisms
Smell and taste	Hydrogen sulphide (H_2S)	Aesthetic inconveniences, toxic effect
Microplastics	Polyethylene, polypropylene and other polymers	Threat to aquatic environment
Endocrine Disrupting Compounds	Bisphenol A, 4-nonylphenol, nonylphenol mono- and di-ethoxylate and others	Ecotoxicological effects on aquatic ecosystem
Pathogenic microorganisms	Pathogenic bacteria, viruses	Public health risk

2.1.3 Organic matter and carbon

All living organisms depend on the supply of necessary elements from the Earth, namely hydrogen, oxygen, carbon, nitrogen and phosphorus. Since the microbes are critical in the process of breaking down and transforming dead organic material into forms that can be reused by other organisms, the microbial enzyme systems involved are viewed as key “engines” that determine the Earth's biogeochemical cycles (Gougoulias et al. 2014). Therefore, organic matter plays a huge role in the environment. Soil organic

matter content affects nutrient retention, water holding capacity and the soil's ability to provide nutrients for plant growth. In the discharged wastewater to a stream, the content of organic matter determines how much oxygen is available for the fish to breathe (Hamilton 2016).

The terrestrial carbon cycle is present in Figure 2.21 and is determined by the balance between photosynthesis and respiration. Initially, carbon is transferred from the atmosphere to soil via "carbon-fixing" autotrophic organisms (photosynthesis), that synthesize atmospheric carbon dioxide into organic material. Then, fixed carbon is returned to the atmosphere through different paths, that include the respiration of animals and plants. The reverse route involves the decomposition of organic material by "organic carbon-consuming" heterotrophic microorganisms, in which they use the carbon of either plant, animal or microbial origin as a substrate for metabolism (in their biomass) (Hamilton 2016).

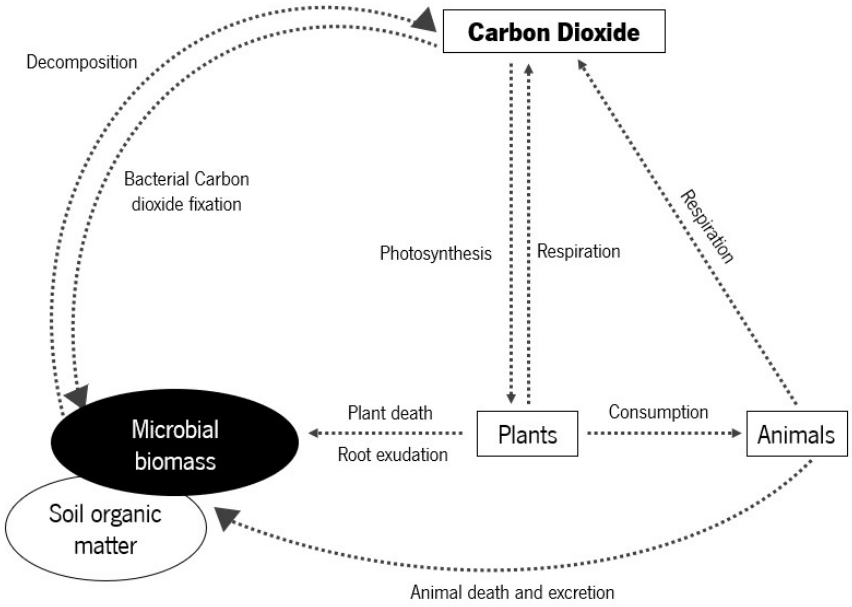


Figure 2.1: The terrestrial carbon cycle with the major processes mediated by soil microorganisms (adapted from Gougoulias *et al.* 2014)

Treatment processes have been developed to remove pollutants in wastewater thereby eliminating their negative effects on human and environmental health. Organic matter has been identified as a pollutant of concern in water and effluents, which affect their treatability (Gursoy-Haksevenler & Arslan-Alaton 2020).

2.1.4 Nitrogen and Phosphorus

Most sources of wastewater contain N and P. If not removed, a build-up of these nutrients in the natural aquatic environments can lead to eutrophication and toxicity. Consequently, there are limits on their discharge to prevent environmental damage (Bouwman 2013; Kube *et al.* 2018). The Water Framework Directive of October 23rd (2000) is the European Union key piece of legislation relating to water. This diploma makes it possible to establish protection measures against contamination and alteration of the state of the water. This protection targets wetlands, aquatic and terrestrial ecosystems (in https://www.apambiente.pt/dqa/index.html). In Portugal, the DL 236/98, of August 1st, establishes the maximum permissible concentrations of 15 mg/L for total N (TN) and 10 mg/L for total P (TP), in general cases (Ministério do Ambiente 1998).

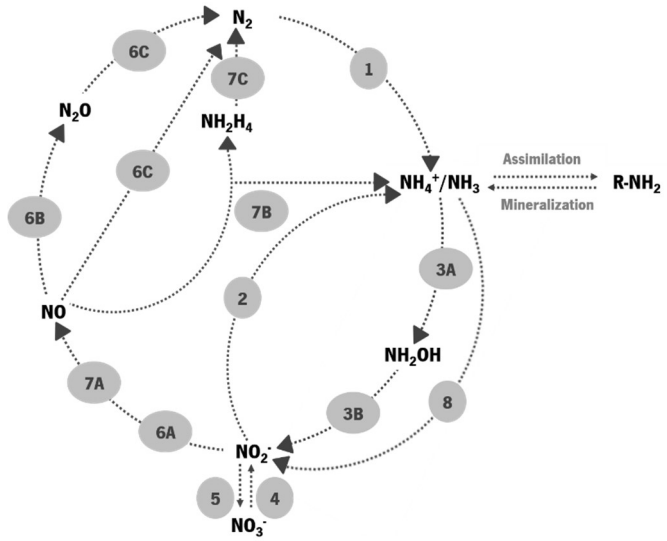
N is essential for all living organisms on Earth as it is a component of many biomolecules, including proteins and nucleic acids in microbial, plant and animal life (Bernhard 2010; Daims *et al.* 2016). N is abundant in the atmosphere as dinitrogen gas (N_2) as well as it is present in earthen and aquatic environments in many different forms (e.g., ammonium ion (NH_4^+), nitrite ion (NO_2^-), nitrate ion (NO_3^-) and amino acids).

Human activities such as fertilizer utilization, burning of fossil fuels, industrial effluents, landfill leachates, have dramatically increased the amount of N wastewaters arriving to WWTP. In order to avoid discharges to the environment causing negative impacts to the biota or public health, they must be properly treated before being discharged (Nourmohammadi *et al.* 2013).

During many years it was assumed that the N biogeochemical cycle consisted in three processes — N_2 fixation, nitrification, and denitrification — and involved microorganisms had historically been characterised by their identified participation in one of these processes, that is, “N fixers”, “nitrifiers” and “denitrifiers” (Stein & Klotz 2016). In the post-genomic era, with a deeper knowledge of the microorganisms and enzymatic processes that transform biologically N, through much-improved instrumentation, a great wealth of data, increased interdisciplinary and international collaboration, the N cycle is represented in a different manner (Sur *et al.* 2010). Figure 2.2 (a) provides the actually accepted N transformation flows: ammonification, including N fixation, and assimilatory and dissimilatory reduction of nitrite (Figure 2.2 (a), reactions 1 and 2); nitrification (Figure 2.2 (a), reactions 3A, 3B and 4); denitrification, including canonical, nitrifier-dependent and methane oxidation-dependent denitrification (Figure 2.2 (a) reactions 6A–D); anammox as a form of coupled nitrification-denitrification (Figure 2.2 (a),

reactions 7A– C);nitrite–nitrate interconversion (Figure 2.2 (a), reactions 4 and 5) and comammox (Figure 2.2 (a), reaction 8). The general processes of organic matter mineralization (often mislabelled as "ammonification") and assimilation (often incorrectly claimed to include processes that regulate the generation of ammonium and its uptake) by cellular life complete the movement of reactive N through the biosphere (Stein & Klotz 2016). Figure 2.2 (b) displays the N cycle intermediates, representing nine oxidation states of N transformation.

(a)



(b)

Molecule	Name		Oxidation State
C-NH_2	Organic - N		Reduced
NH_3, NH_4^+	Ammonia, Ammonium	-3	
N_2N_4	Hydrazine	-2	More electrons
NH_2OH	Hydrolylamine	-1	
N_2	Dinitrogen	0	
N_2O	Nitrous oxide	+1	
NO	Nitric oxide	+2	
HNO_2, NO_2^-	Nitrous acid, Nitrite	+3	Fewer electrons
NO_2,	Nitrogen dioxide	+4	
HNO_3, NO_3^-	Nitric acid, Nitrate	+5	Oxidized

Figure 2.2: (a) Major processes of the N cycle (adapted from Stein & Klotz, 2016). Reactions that comprise the major processes of the N cycle are represented by the numbered circles: ammonification may be accomplished either by process **1** (N fixation) or by process **2** (dissimilatory nitrite reduction to ammonium); nitrification is composed of process **3** (3A,3B) (oxidation of ammonia to nitrite) and process **4** (oxidation of nitrite to nitrate); process **5**, reduction of nitrate to nitrite, can be coupled to processes **2**, **6** or **7** in a population or a community; denitrification is shown as process **6** (6A,6B,6C,6D) (nitrifier-dependent and methane oxidation-dependent denitrification); anammox is displayed as process **7** (7A,7B,7C) (coupled nitrification-denitrification); process **8**, oxidation of ammonia to nitrate, is named as comammox. Organic matter mineralization to ammonia completes the N cycle. **(b)** N cycle intermediates, representing nine oxidation states (that donate or accept electrons), are shown in the table.

N fixation is accomplished by few genera of bacteria (e.g. *Azotobacter*), archaea, and blue-green algae (*Cyanobacteria*), which encode nitrogenase enzyme complexes (Sadik *et al.* 2016; Stein & Klotz 2016). The ammonium produced by N fixation is either assimilated into biomass or is further respired by aerobic and anaerobic ammonia-oxidizing microorganisms.

Nitrification is the process where specific microorganisms convert ammonia to nitrite and nitrite then to nitrate. The process of nitrification involves three groups of microorganisms: **(1)** Ammonia oxidizers oxidize ammonia to nitrite and include bacteria of the genus *Nitrosomonas* (e.g., *N. europaea, N. oligocarbogenes*) as well as *Nitrosospira*, *Nitrosococcus*, *Nitrosolobus*, and *Nitrosovibrio* (Fumasoli *et al.* 2017) and ammonia oxidizing Archaea (Könneke *et al.* 2005); **(2)** Nitrite oxidizers convert nitrite to

nitrate and include *Nitrobacter* spp. (e.g., *N. vulgaris*, *N. winogradski*) as well as *Nitrospira*, and *Nitrococcus* (Bitton 2005); **(3)** Complete ammonia oxidizers oxidize ammonia all the way to nitrate (comammox) and include *Nitrospira* (Daims *et al.* 2015).

Denitrification describes the process of anaerobic respiration of nitrate to nitrite, nitric oxide (NO), and nitrous oxide (N_2O) to N_2 and is carried out by a diverse group of microorganisms such as of the genera *Thiobacillus* (*Thiobacillus denitrificans*), *Alcaligenes* and *Pseudomonas*, (e.g., *P. fluorescens, P. aeruginosa)*, among others (e.g., *Paracoccus* sp.) (Bitton 2005; Gerardi 2006). Recently, many microorganisms such as *Alcaligenes faecalis*, *Bacillus* sp. or *Rhodococuus pyridinivorans* have been found to conduct both heterotrophic nitrification and aerobic denitrification. These microorganisms have advantages such as procedural simplicity, where nitrification and denitrification can take place simultaneously or with less acclimation problems (Shoda & Ishikawa 2014; He *et al.* 2016).

Anammox process (anaerobic ammonia oxidation) is carried out by prokaryotes belonging to the Planctomycetes phylum of Bacteria. *Brocadia anammoxidans* was the first described anammox bacterium. Anammox bacteria oxidize ammonia by using nitrite as the electron acceptor to produce gaseous nitrogen (Daims *et al.* 2015).

Phosphorous (P) is also an essential component of Deoxyribonucleic acid (DNA), amino-acids, molecules that store energy (Adenosine diphosphate (ADP) and Adenosine triphosphate (ATP)) and lipids of cell membranes. P in a wastewater treatment system can be classified as soluble P and particulate P. Soluble P can be divided into soluble phosphate, soluble polyphosphate and soluble organic phosphate. Usually, the discharge criteria are based on the TP concentration including both dissolved and particulate P (Li & Brett 2015; Park *et al.* 2016; Ge *et al.* 2018).

The major transformations of P in aquatic environments are described in Figure 2.3.

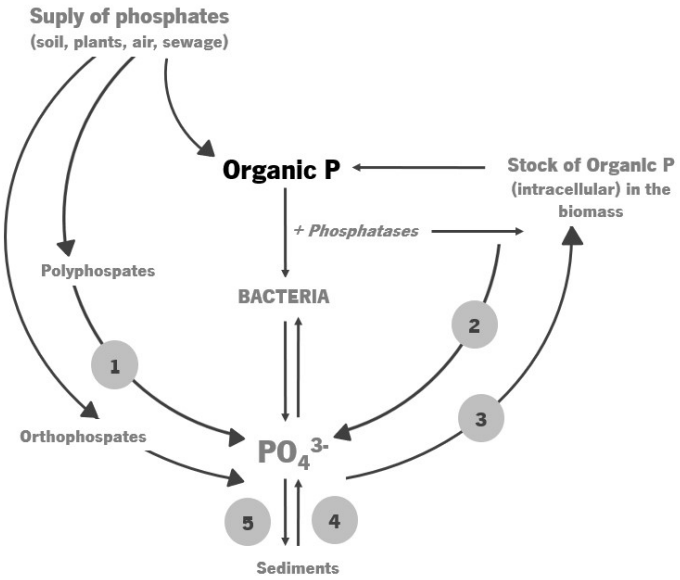


Figure 2.3: Major P transformation processes in the aquatic environment (adapted from https://www.suezwaterhandbook.fr/eau-et-generalites/quelles-eaux-a-traiter-pourquoi/les-eaux-naturelles/cycle-du-phosphore and Ramothokang *et al.* 2009). Reactions that comprise the major processes of the P transformation are represented by the numbered circles: hydrolysis (into the ecosystem) is composed of process **1**; excretion (from animals) by process **2;** process **3** represent the assimilation of dissolved organic P and process **4** and **5** represent the aerobic and anaerobic P cross flows between water and sediments, respectively.

Orthophosphate ion (from supply of phosphates) is one of the predominant inorganic forms of P and is directly absorbed by microorganisms (Ramothokang *et al.* 2009; Metcalf & Eddy 2013). Organic compounds are converted to orthophosphate by a wide range of microorganisms such as bacteria (*Bacillus subtilis* and *Arthrobacter* sp.) and fungi (*Aspergillus* sp., *Penicillium* sp.) (Saxena 2015; Panpatte *et al.* 2017). The P compounds are used by microorganisms and integrated in the composition of several macromolecules in the cell. An important transformation of P is called as precipitation; when it occurs, the orthophosphates, whose solubility is controlled by pH and by the presence of ions of calcium (Ca^{2+}), magnesium (Mg^{2+}), ferric iron (Fe^{3+}) and aluminium (Al^{3+}), are converted to insoluble compounds (e.g.: Calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)) (Bitton 2005). The insoluble compounds are used in the production of organic and inorganic acids (through the metabolic activity of microorganisms) leading to the liberation of orthophosphates.

2.2 Activated-sludge systems

The activated-sludge process was developed in England in 1914, by E. Ardern and W.T. Lockett and is the most commonly used technology for biological wastewater treatment. The success of any activated-sludge wastewater process depends on the metabolic activity and morphological characteristics of the microbial population in suspension and hence, on the ability of the microbial biomass to settle out of the

effluent stream after removal of soluble organic matter (de los Reyes *et al.* 1997). Currently, there are several versions of the original activated-sludge process, an effort to maximise process efficiency and enhance the importance of microbial communities as an integral component of these biological treatment systems (Valentín-Vargas *et al.* 2012). Despite the design modifications, its elementary mode of operation is very similar and based on two basic stages (Figure 2.4). In the biochemical stage (aeration tank), the organic matter (influent) is introduced into a reactor where an aerobic bacterial community is kept in suspension, forming the mixed liquor. In the physical stage (secondary clarifier), the biological suspended solids from mixed liquor (MLSS) are removed from the process flow stream, resulting in an effluent low in suspended solids, to eventually undergo further treatment before discharge. The settled solids return to the aeration tank and the excess solids are purged to ensure a desired MLSS concentration in the aeration tank.

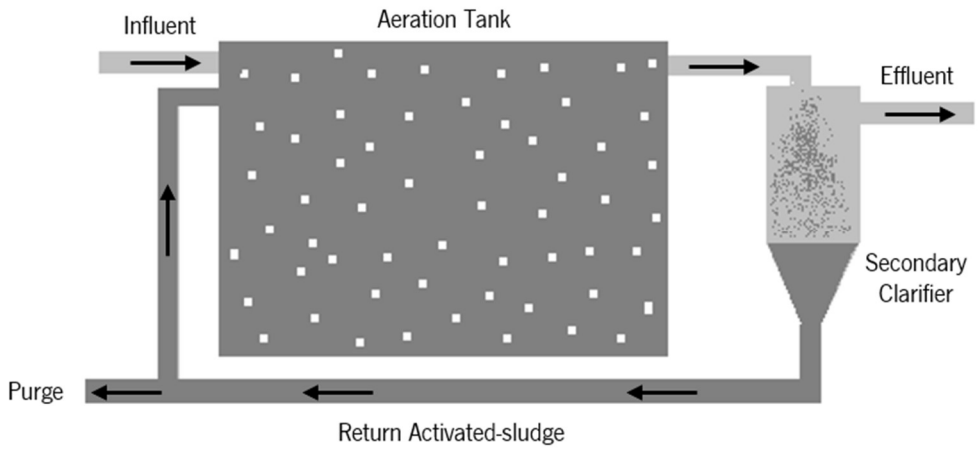


Figure 2.4: Conventional activated-sludge process.

In the aeration tank, the influent is mixed with recirculated sludge (settled biosolids) thus forming the mixed liquor, containing typically about 1500 to 2500 mg/L of suspended solids (Bitton 2005).The oxidation of organic matter under aerobic conditions should be assured under 1 to 2 mg O_2/L (Jenkins *et al.* 2004; Haandel & Lubbe 2012; Metcalf & Eddy 2013). Influent organic matter is oxidized by microorganisms and the obtained energy is channelled to vital processes, being the other part converted to new biomass. The recirculation of large portion of the activated-sludge causes a higher residence time of solids (SRT) and hydraulic retention time (HRT). This allows keeping inside the aeration tank the microorganisms that oxidize the organic compounds.

Therefore, in this process, some important factors are monitored, such as the physical-chemical variables (e.g. biological oxygen demand (BOD) and chemical oxygen demand (COD), pH, TN, TP and feeding regime) and the operating conditions (e.g. MLSS, sludge return rate, DO and the hydraulic load).

2.2.1 Microorganisms in Activated-sludge

The microbial diversity in this complex ecosystem comprises bacteria, viruses, algae, fungi, protozoa and small metazoa. In this community, bacteria (classically considered decomposers) - which are believed to be about 95 % of the weight of the activated-sludge biomass - play a key role in the conversion of the organic matter and in the removal of nutrients from wastewater (Signorile *et al.* 2010; Amanatidou *et al.* 2016). The consumers, protozoa and small metazoa, feed upon the populations of dispersed bacteria or among themselves (Madoni 1994). The aeration basins develop their own trophic network, in which several populations compete for food, leading to competitive relationships and predation which creates oscillations and successions of the populations until a dynamic equilibrium is attained. This relationship depends greatly on the design and management of the plant, whose maximum goal is to achieve greater purification efficiency.

Ideally, biomass grows in flocs and the bacterial populations of activated-sludge can be divided into floc-forming and non-floc-forming (mostly filamentous bacteria). The floc-forming bacteria initiate the floc formation and are essential for its success (Arelli *et al.* 2009). In this case, a large and diverse population of bacteria are packaged in numerous floc particles. Floc formation occurs the increase of sludge age, allowing the production of the necessary cellular components to stick together or agglutinate (Table 2.2).

Table 2.2: Genera of floc-forming bacteria found in activated-sludge process (adapted from Gerardi, 2006)

Floc-forming Bacteria
Achromobacter
Citromonas
Aerobacter
Escherichia
Alcaligenes
Flavobacterium
Arthrobacter
Pseudomonas
Bacillus
Zoogloea

Therefore, the floc (Figure 2.5) is a heterogeneous structure composed by aggregates of bacteria and other organisms, as well as organic and inorganic particles, immersed in a matrix or gel, formed by extracellular polymeric substances (EPS) (Seviour & Nielsen 2010; Wágner *et al.* 2015). A second type of bonding process arises when the network formed by filamentous bacteria (non-floc-formers) serves as a free surface where floc-formers can attach. It is imperative that the proportion between filamentous and floc-forming bacteria is well-adjusted in order to allow the build-up of large (10-2000 μm) irregular, dense and strong flocs, creating well- settling sludge (Wágner *et al.* 2015).

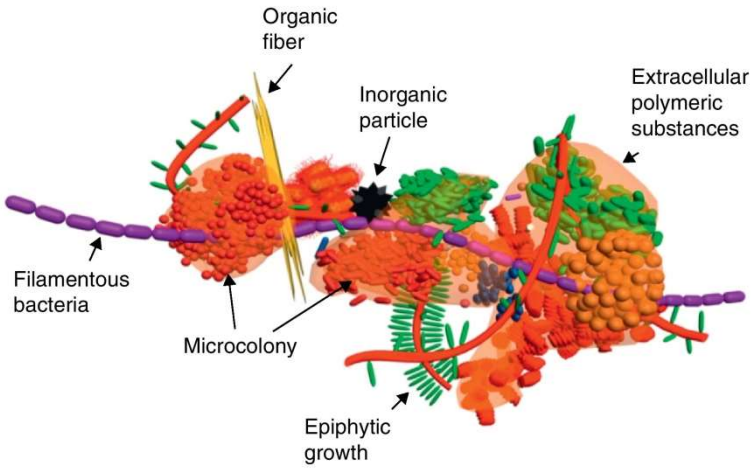


Figure 2.5: Schematic structure of an activated-sludge floc (from Seviour & Nielsen, 2010).

The floc macrostructure reveals the amount and organization of the filamentous bacteria where the floc organization itself allows gravity sedimentation, which is a principal monitoring parameter in WWTP

management (Stalder *et al.* 2013). The floc morphology can be characterised in several ways reflecting differences in their shape, firmness, compactness and size (Jenkins *et al.* 2004; Seviour & Nielsen 2010):

a. the ideal floc, where the filaments are mostly restricted to the interior of the floc, being barely seen in the bulk liquid;

b. pinpoint flocs with little or no filamentous matrix. These flocs are small and fragile, and very easily disrupted by shear or turbulence. The small floc fragments settle poorly, originating a turbid effluent;

c. filamentous bulking sludge, with internal extensive growth of filamentous bacteria and significantly extending to the outside of the floc. These filaments make the flocs diffuse and often provide floc-to-floc bridging, both leading to poor settling ability and compaction.

The performance of the activated-sludge process is largely dictated by the ability of the secondary clarifier to separate and concentrate the biomass from the treated effluent. Since the effluent from the secondary clarifier is most often not treated any further, a good separation in the settler is critical for the whole plant to meet the effluent standards (Banadda *et al.* 2011). The poor separation and thickening of activated-sludge in secondary clarifiers can lead to operation problems (Table 2.3).

Table 2.3: Problems associated with inefficient separation of biomass in secondary clarifiers (adapted from Tandoi *et al.*, 2017)

Operational consequence
Activated-sludge biomass escapes from secondary clarifier and deteriorates final effluent quality not only in terms of suspended solids, but also in five-day biochemical oxygen demand (BOD_5) (respiration of biomass), COD (organic mature of biomass) and TP (the biomass content of N and P)
A dilute return sludge stream is produced that my not allow maintenance of a desire aeration basin biomass concentration and proper control of sludge age
A dilute waste sludge stream hydraulically overloads the sludge handling facilities

The growth of activated-sludge bacteria in the form of filaments is not a response to certain environmental conditions but rather an intrinsic characteristic of certain species. Eikelboom (1975) described 29 different filamentous bacteria, based only on morphology and clustered them in three groups: (1) bacteria referable to known genus and species; (2) microorganisms that have a name but lack description of, for instance, phenotypic data; and (3) microorganisms to which it was impossible to attribute a biological identity and are named as "Type" followed by a number. The most common filamentous bacteria found in activated-sludge processes are presented in Table 2.4.

Table 2.4: Common filamentous bacteria in activated-sludge (adapted from Gerardi, 2006)

Filamentous Bacteria	
Beggiatoa	Type 0581
Haliscomenobacter hydrossis	Type 0675
Microthrix parvicella	Type 0803
Nocardioforms	Type 0961
Nostocoida limicola	Type 1701
Sphaerotilus natans	Type 1702
Thiothrix	Type 1851
Type 0041	Type 1863
Type 0092	Type 021N

In an ecosystem where bacteria predominate over all other groups involved in wastewater depuration, the importance of the presence of protozoa and small metazoa (like rotifers) is of relatively recent perception (Madoni 1994). These eukaryotic organisms have the ability to feed on organic particles, such as suspended bacteria, so it is generally assumed that their main role in activated-sludge systems is the clarification of the effluent. They can occur in very large numbers and commonly reach densities of about 10^7 cells/L in the aeration tank (Nicolau *et al.* 2001). Concentrations below 10^5 cells/L are indicative of low efficiency of the system (Sibewu *et al.* 2008). In activated-sludge systems, protozoa are separated in five functional groups based primarily on motility and feed behaviour: amoebae, flagellates, free-swimming ciliates, crawling ciliates and stalked ciliates (or attached ciliates) (Ginoris *et al.* 2007).

The ciliates usually dominate the protozoan community, and for that reason, along with the fact of being sensitive to environmental conditions, they are generally the first to be considered as *in situ* bioindicators of the process (Dubber & Gray 2009; Hu *et al.* 2013; Hailei *et al.* 2017). The first sign of an adverse environmental condition is generally the slowing or cessation of cilia movement. Simultaneously, the number of flagellates and free-swimming ciliates increases and they eventually dominate the aeration tank. This is an indication of the breakup of the floc and an overabundance of dispersed bacteria used by these organisms as a source of food. In severe cases, these protozoa die and can contribute to the formation of foam (Jenkins *et al.* 2004).

The optimal scenario in activated-sludge systems occurs when there is a balance between free-swimming and stalked and crawling ciliates and rotifers. The dominance of flagellates, amoebas or free-swimming ciliates indicates the presence of high organic load and thus dispersed bacteria in the system, but the dominance of attached ciliates, crawlers and rotifers indicates the opposite: the bacteria are mainly

aggregated and only highly efficient bacteria grazers, as the attached ciliates and the rotifers, can survive in high densities; the crawlers, grazing on the loose attached bacteria, can grow in their specific ecological niche. Some WWTP attempt to adjust the process parameters based on the protozoa community, since studies focused on the relationships between protozoa and physical-chemical and operational parameters have revealed that the species structure of these communities could be an indicator of plant efficiency (Madoni 2011). The microfauna of an efficient activated-sludge system usually presents the following characteristics (Madoni 1994):

- High population density of microfauna (≥ 10^6/L);
- Community composed mainly of crawling and attached ciliates, with the flagellates practically absent;
- Diversified community where no species or group dominates numerically by more than a factor of 10.

When a particular group dominates the aeration tank, conclusions can be drawn on the expected performance or the possible causes of malfunctioning of the system (Table 2.5).

Table 2.5: Possible relationship between presence of particular dominant groups of protozoa, plant performance and operational characteristics (adapted from Madoni, 1994)

Dominant Protozoa	Performance	Possible causes
Small flagellates (except choanoflagellates)	Poor	Poorly aerated sludge, overloaded plant
Small flagellates and small naked amoebae	Poor	High load and/or hardly degradable load
Small free-swimming ciliates	Mediocre	Poorly aerated sludge, low sludge age
Large free-swimming ciliates	Mediocre	Overloaded plant
Certain attached ciliates (i.e., *Opercularia* spp. and *Vorticella microstoma*)	Failing	Transient perturbations
Crawling ciliates	Good	-
Attached and crawling ciliates	Good	-
Testate amoebae	Good	Low or diluted load, good nitrification

The routine analysis of the microfauna as an indicator of the performance of the activated-sludge processes has become increasingly common as it provides very useful information on the biological activity of sludge, based on the structure of the microbial community (Jiang & Shen 2005). A significant breakthrough came up when Madoni, in 1994, developed an objective index - the Sludge Biotic Index (SBI) (Madoni 1994), widely used in activated sludge monitoring since then. He proposed that this index

would allow the biological quality of the sludge to be expressed objectively in numerical terms, categorizing mixed liquors into four classes based on their SBI value (Table 2.6). Essentially, the method is based on two principles: (1) the dominance of the microfauna key groups changes with environmental and operational conditions of the plant; (2) the number of morphological species is reduced as the plant performance deteriorates. However, the SBI only evaluates the operation of the plant in what the aeration tank is concerned and does not allow to predict the quality of the treatment in the secondary clarifier, even when any problems at this level give rise to variations in the microfauna structure. The known correlations between the microfauna groups and the main physico-chemical and operational parameters allow the selection and grouping of microfauna organisms into positive and negative key groups. Positive key groups are crawling ciliates, stalked ciliates, and amoebae; negative key groups comprise flagellates, free-swimming ciliates, and the attached ciliates *Vorticella microstoma*, *V. infusionum* and *Opercularia* spp. (Madoni 2011).

Table 2.6: Conversion of SBI values into four activated-sludge quality classes (adapted from Madoni, 1994)

SBI value	Class	Judgement
8-10	I	Very well colonized and stable sludge, excellent biological activity; very good performance
6-7	II	Well colonized and stable sludge, biological activity on decrease; good performance
4-5	III	Insufficient biological depuration in the aeration tank; mediocre performance
0-3	IV	Poor biological depuration in the aeration tank; low performance

With the evolution of wastewater treatment systems or the presence of new compounds in the wastewater, doubts about the applicability of SBI to these new WWTP designs raised among the research community. Drzewicki & Kulikowska (2011) reported that the reduction in wastewater treatment performance of septic tanks was not reflected by SBI values, i.e., despite a decrease in WWTP performance, SBI values could remain high. Arévalo *et al.* (2009), Papadimitriou *et al.* (2004) and Samaras *et al.* (2009) also suggested that changes in the abundance of some protozoa could indicate, better than the SBI calculation, the performance level of the WWTP. Thus, it is important to consider specific protozoan species as indicators of plant performance especially when certain WWTP configurations are considered (Araújo dos Santos *et al.* 2014).

2.2.2 Identification of activated-sludge microorganisms

As can be inferred from what has been mentioned before, the identification of microorganisms inhabiting the mixed liquor is an extremely useful tool to diagnose and correct WWTP malfunctions.

Remarkably, the isolation and identification of filamentous bacteria from activated-sludge initially attracted little attention from microbiologists mainly due to the fact that these identifications required a lot of work and self-expertise. Identification of filamentous bacteria by conventional methods involves isolation to obtain pure cultures, followed by complex characterization tests. These procedures are therefore not appropriate for biodiversity study of a natural or engineered ecosystem (Sanz & Köchling 2007). Lack of pure cultures often means that several filamentous bacteria in activated-sludge cannot be identified and cannot be taxonomically recognized or provided with a name, and therefore, they are not documented in the standard microbiological identification manuals (Speirs *et al.* 2009).

To characterise a wide set of samples of activated-sludge in terms of prevalence, dominance, and abundance of diverse morphotypes, including different sub-types, wet mounts are prepared to be further observed under phase-contrast microscopy. Smear mounts must also be prepared and stained by Gram and Neisser techniques to be further observed under bright-field microscopy. This, along with the examination of some morphological characteristics, can provide a preliminary identification of these bacteria. This identification can be made by the different morphological characteristic such as motility, branching and diameter of the filament, shape of the cells or the filament and, if existing, attached growth, septa, sheath and granules (Table 2.7).

Table 2.7: Morphological characters used in identifying filamentous bacteria in activated-sludge (adapted from Seviour & Nielsen, 2010)

Cells	Descriptions
Dimensions	Length and diameter
Shape	Rods, cocci, discs
Septa	Presence or absence
Inclusions	Presence of polyphosphate, poly-β-hydroxyalkanoates or sulphur granules
Motility	By gliding or possession of flagella
Thichomes	Descriptions
Dimensions	Length and diameter
Shape	Straight, bent or coiled
Location	Within floc or free in bulk liquid
Attached growth	Presence or absence of attached bacterial cells
Branching	True or false branching
Sheath	Presence or absence
Staining reactions	Staining reactions
Gram stain	Positive (purple) or negative (red)
Neisser stain	Positive (mauve) or negative (brown)

Eikelboom (1975) developed the first dichotomic key to identify filamentous bacteria in activated-sludge systems, mostly based on morphological characteristics and on the response of the filamentous bacteria to a few microscopic staining tests. The procedures, techniques and identification keys were compiled in a manual (Eikelboom 2000) that which together with the manual by Jenkins *et al*. (2004), has been used as world-wide reference on filamentous bacteria identification. Figure 2.6 outlines the identification key of filamentous bacteria in activated-sludge.

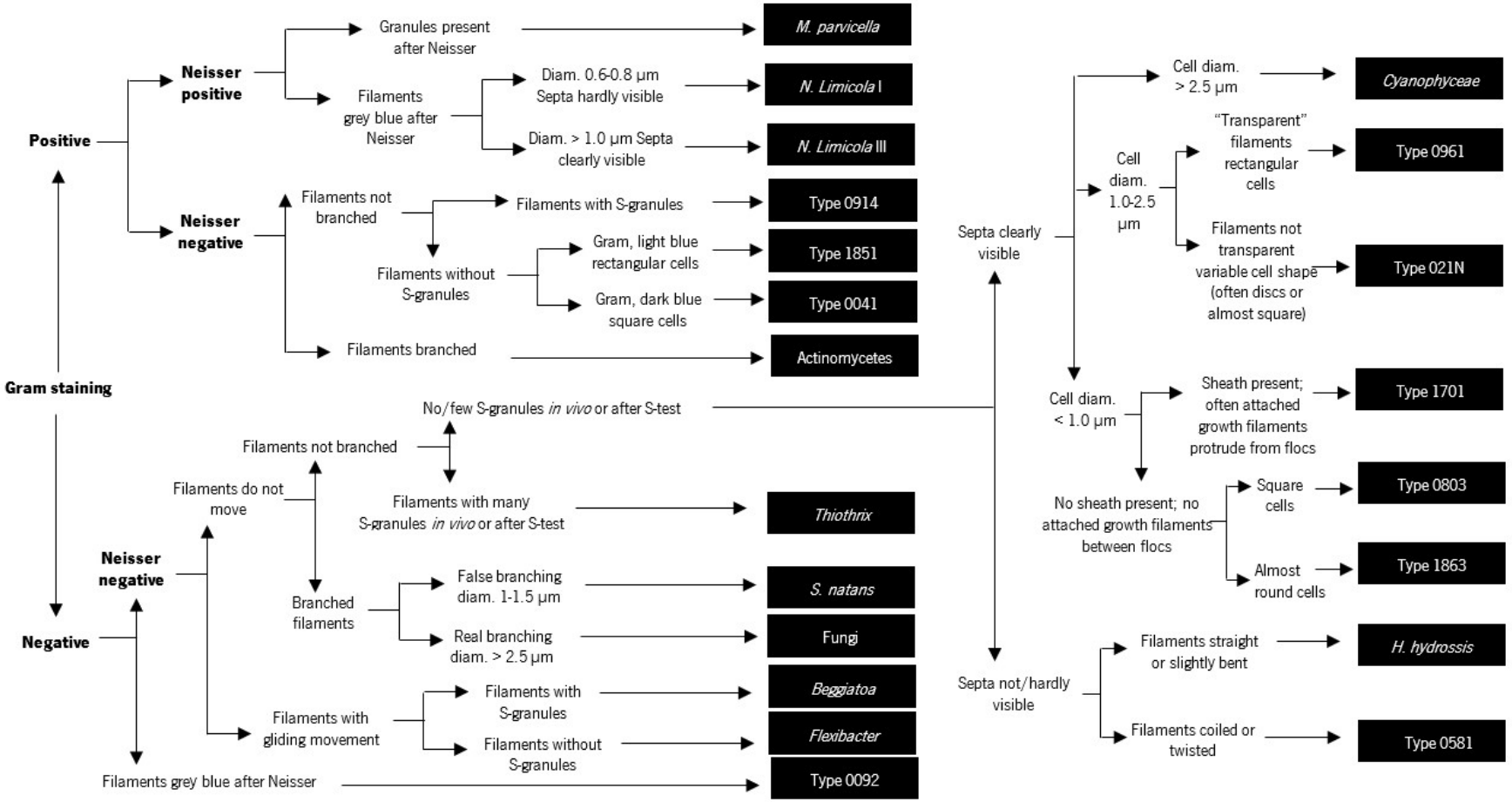


Figure 2.6: Identification key of filamentous bacteria in activated-sludge systems (adapted from Eikelboom, 2000).

Although filamentous bacteria can be classified in many ways, the most important evaluation of these bacteria for WWTP operators lies on the roles (positive and negative) that bacteria perform, being these roles attributed according to their relative abundances (Gerardi 2006). Table 2.8 presents the relative abundance scores of filamentous bacteria in activated-sludge process, as determined by Jenkins *et al.* (2004).

Table 2.8: Relative abundance scores for filamentous organisms in mixed liquor (adapted from Jenkins *et al.*, 2004)

Score value	Assessment of filamentous abundance	Comments
0	None seen	-
1	Few present	Only seen in an occasional floc
2	Some present	Commonly seen but not in all flocs
3	Common	Seen in all flocs but only at low frequencies (1-5 filaments per floc)
4	Very common	Seen in all flocs at frequencies of 5-20 per floc
5	Abundant presence	Seen in all flocs at frequencies of ≥ 20 per floc
6	Excessive numbers	Seen in all flocs. Biomass mostly filaments which are plentiful in the bulk liquid

When the relative abundance of filamentous bacteria belongs to the categories from "none seen" (0) to "some present" (2), no positive role is attributed to the respective species or morphotype as it is not present in significant numbers. The positive role is only experienced if the score of filamentous bacteria is "common" (3). The negative role begins at the level of "very common" (4) to "excessive numbers" (6), mainly due to the resulting settleability problems, loss of solids, and bulking/foam production.

Conventional microscopic sludge examination is a crucial monitoring instrument, since it provides information about sludge quality and operation conditions and also the detection of filamentous bacteria (Müller *et al.* 2007). Despite its usefulness, microscopic identification (conventional microscopy) has some limitations (Adonadaga & Martienssen 2016): several filamentous bacteria (e.g., the morphotypes *S. natans*, Type 1701, Type 0092 and Type 0961) can modify their morphology in presence of different environmental conditions (Buali & Horan 1989; Seviour & Blackall 1999) and, regardless physiological and taxonomic differences, some of them have similar appearance (Martins 2004). Figure 2.7 displays some advantages and disadvantages of microscopic identification methods for the identification of filamentous bacteria.

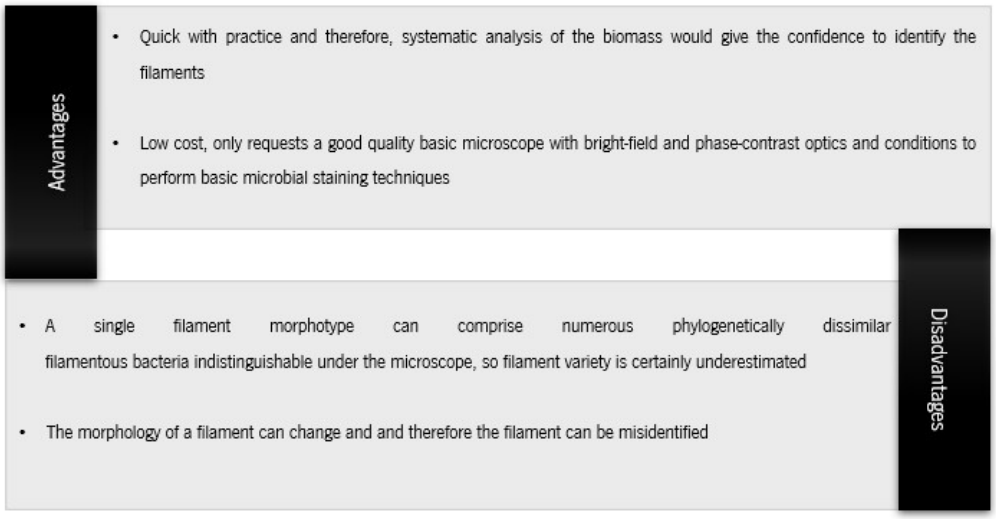


Figure 2.7: Advantages and disadvantages of filamentous bacteria conventional identification.

Misleading and hard identification of filamentous bacteria by traditional microscopic techniques directed research towards molecular methods (Santos *et al.* 2009). Among the techniques used, cloning and the creation of a gene library, denaturant gradient gel electrophoresis (DGGE), fluorescence *in situ* hybridization (FISH) and microautoradiography (MAR) associated with FISH, stand out (Sanz & Köchling 2007). Nevertheless, these techniques require expensive equipment and skilled personnel, that is not so simple to implement in a routine way in WWTP.

Concerning protozoa and small metazoan in activated-sludge, their microscopic observation still is a common and widespread practice because it is accepted that the presence or absence of a specific species, the dominance of certain species and the composition of the microfauna may generally be considered good indicators of the biological purification efficiency of the activated-sludge system. Furthermore, protozoa and small metazoa are relatively easy to observe and identify (at least to the taxonomic categories needed for the monitoring of the process) with a normal optical microscope. It is usually recommended to follow this procedure frequently, several times a week or even daily, because protozoan community can change rapidly (Jenkins *et al.* 2004).

2.3 Filamentous bulking

Serious problems of filamentous bulking and foaming are worldwide known to cause a variety of operational problems in activated-sludge plants. Moreover, they may lead to environmental problems due to the release of large amounts of untreated effluent and can represent even a public health drawback

because of the potential spread of pathogens, namely through the spread of aerosols. It is calculated that these incidents lead to significant economic losses, related to direct and indirect effects.

In the past decades, some surveys conducted in The Netherlands (Eikelboom 1977), Germany (Wagner 1982) , United States of America (USA) (Strom & Jenkins 1984), South Africa (Blackbeard *et al.* 1986), Australia (Seviour *et al.* 1990), France (Pujol *et al.* 1991), Denmark (Holm Kristensen *et al.* 1994), Thailand (Mino 1995), Czech-Republic (Wanner *et al.* 1998), Italy (Madoni *et al.* 2000), Argentina (Di Marzio 2002) and Japan (Martins 2004) have revealed the extent and severity of both bulking and foaming phenomena. In Portugal, several cases of bulking and foaming were studied along a 2-year sampling study and the causes identified (Neto *et al.* 2008). These phenomena have drawn considerable interest to both engineers and microbiologists and several works discuss in detail the reasons for their excessive filament proliferation (Eikelboom 2000; Jenkins *et al.* 2004; Martins *et al.* 2004; Nielsen *et al.* 2009; Seviour & Nielsen 2010; Tandoi *et al.* 2017).

In addition to undesired filamentous bacterial growth (overabundance), there are two filamentous bacteria/floc particle morphologies that characterise the filament bulking and contribute to settleability problems (interfering) and loss of solids (Cao & Lou 2016). One of these morphologies is interfloc bridging, which happens when bacteria extend their filaments from the flocs and a connection is set up between two or more floc particles. The other is the open floc formation which results in the scattering of the floc bacteria in small groups along the lengths of the filamentous bacteria in the floc particle, thus producing a diffuse (or stretched-out) floc structure (Gerardi 2006; Nittami *et al.* 2017). There are several types of activated-sludge solids separation problems. These problems are usually classified in terms of the effects that they cause in the treatment process and therefore their definitions are not always accurate (Jenkins *et al.* 2004). Jenkins *et al.* (2004) summarized the causes and effects for poor solids separation and some are not caused by overgrowth of filamentous bacteria (Table 2.9).

Table 2.9: Problems associated with separation of biomass in activated-sludge process (adapted from Jenkins *et al.*, 2004)

Problem	Origin	Result
Dispersed growth	No formation of flocs; microbial single cells	Effluent is turbid and solids do not settle
Non-filamentous or Zoogloeal bulking	Microbial over-production of exopolysaccharide. Associated with industrial effluents with deficient N or P	Reduced settling and compaction rates; solids carry over from clarifier
Pinpoint floc	Small, compact and weak flocs. Smaller flocs settle slowly and larger settle rapidly. Operating at low Food/Microorganism ratios (F/M)	Turbid effluent; Low sludge volume index (SVI)
Filamentous bulking	Filamentous bacteria extend from flocs, bridging and open floc occurs interfering with compaction, settling and thickening	High SVI and, solids overflow from clarifiers
Blanket rising	Denitrification in the clarifiers, the N_2 gas produced attaches to the flocs, which float to the surface. Intensified by long retention time of solids in the clarifiers	Surface of clarifiers covered with biomass
Foaming or scumming	Caused mainly by hydrophobic bacteria like Nocardioforms, Candidatus "*M. parvicella*" and other actinobacteria or Type 1863	Foams can float large amounts of biomass to surface of clarifiers

In municipal activated-sludge plants, 30 different filamentous bacteria morphotypes were identified (Eikelboom 2000; Jenkins *et al.* 2004; Wang *et al.* 2016) and other 40 morphotypes have seen described in industrial plants (Eikelboom & Geurkink 2002). Filamentous bacteria morphotypes and their abundance appear to be different in the cases where N removal with nitrification-denitrification stages exist. Eikelboom & Geurkink (2002) consider that the nutritional supplies have more influence in the abundance and the type of filamentous than the geographical location, and consequently the filamentous bacteria ranking order in different countries is mainly a response to variations in the treatment plant design, operation and influent wastewater characteristics (Seviour & Nielsen 2010; Wang *et al.* 2016). On the other hand, Mielczarek *et al.* (2012) investigated over 30 full-scale WWTP with nutrient removal to identify and quantify the dominant filamentous bacteria through FISH, describing their population dynamics and relating the presence of specific filamentous bacteria to treatment plant design, process parameters and wastewater composition. With this study, they concluded that the composition of the filamentous community was unique to each plant, giving a characteristic "fingerprint", and no significant correlations were found between the presence and abundance of filamentous bacteria and plant design or process parameters. More considerations on this matter are presented in section 2.3.1.

As said before, bulking phenomena tends to occur when there is a reduction of sedimentation velocity and, simultaneously, a lower compaction of the sludge in the secondary clarifier, leading to a decrease in the final effluent quality. The filamentous overgrowth can result in (Tandoi *et al.* 2017):

- Low recycle and wasted activated-sludge solids concentrations;
- Poor sludge dewaterability;
- Hydraulic overloading of sludge treatment facilities.

It is possible to make bulking diagnosis by determining the SVI, but the microscopic observation of the sludge also allows to conclude whether it is caused by excessive growth of filaments and, in this case, to the identification of the microorganism in question. A decrease in the floc sedimentation results in an increase in the SVI. If the SVI is above 150 mL/g, filamentous bulking is most probably occurring (Metcalf & Eddy 2013). Generally, a sludge has very good settling characteristics if the SVI value is below 80 mL/g (Liu & Liu 2006). A great diversity of microorganisms can yield filamentous bulking and, as such, a variety of causes might underlie this phenomenon. The most dominant filamentous bacteria in bulking and foaming phenomena in municipal activated-sludge treatment plants according to Seviour & Nielsen (2010) are listed in Table 2.10.

Table 2.10: Ranking of dominant filamentous bacteria in bulking and foaming phenomena in municipal activated-sludge treatment plants (adapted from Seviour & Nielsen, 2010)

Filamentous bacteria	Order of prevalence											
	The Netherlands		Germany	USA	South Africa	Australia	France	Denmark	Thailand	Czech Republic	Italy	Argentina	Japan
Nocardioforms	-	-	-	1	6	7		-	*	6	2	3	*
Type 1701	5	-	8	2	8	12		-	*	13	9	2	
Type 021N	2	4	1	3	-	1		3	*	7	6	1	*
Type 0041/0675	6	2	3	4	6	2	*	2	*	5	3	2	*
Thiothrix spp.	9	-	-	5	9	13		-		8	7	2	*
S. natans	7	5	4	6	-	14		-		-	-	1	*
M. parvicella	1	1	2	7	2	1	*	1		1	1	2	
Type 0092	4	6	-	8	1	3	*	3	*	3	4	3	
H. hydrossis	3	-	6	9	9	5		-		11	-	-	
Type 0803	7	-	10	11	7	9		-		4	-	-	
N. limicola I, II, III	11	-	7	12	8	5	*	-		2	5	-	
Type 1851	12	3	-	13	3	8		6		-	-	-	
Type 0961	10	-	9	14	-	10		-		9	8	-	
Beggiatoa spp.	8	-	-	16	-	15		-		-	-	-	
Type 0914	-	-	-	18	5	7		5		10	10	-	

* Not quantified

The Netherlands (200 WWTP); Germany (315 WWTP); USA (270 WWTP); South Africa (111 WWTP); Australia (65 WWTP); France (12 WWTP); Denmark (38 WWTP); Thailand (6 WWTP); Czech Republic (86 WWTP); Italy (40 WWTP); Argentina (10 WWTP); and Japan (not reported WWTP number)

The most abundant filamentous bacteria in activated-sludge treating municipal and industrial wastewater are summarized in **Erro! Autorreferência de marcador inválida.**. This identification was done by FISH surveys (Seviour & Nielsen 2010).

Table 2.11: Most abundant filamentous bacteria in activated-sludge treating municipal and industrial wastewater (adapted from Seviour & Nielsen, 2010)

Filamentous bacteria	Municipal	Industrial
Alphaproteobacteria (*N. limicola*)		1
Betaproteobacteria (Type 0803)	7	
Thiothrix eikelboomi (Type 021N)		2
Thiothrix types I, II		2
Bacteroidetes (Type 0092)	6	
H. hydrossis	4	
Chloroflexi (Type 1851, Type 0041/0675 and Type 1701)	2	4
TM7-related (Type 0041/0675) *Curvibacter*- related bacteria (Type 1701)	3	
Candidatus *M. parvicella*	1	
Mycolata (Nocardioforms)	5	3

* Rankings are based on all available FISH surveys from Danish, German, Dutch and Italian industrial and municipal WWTP

The filamentous foaming occurrence is characterised by the production of stable foams, which may give rise to problems either by their exit in the final effluent or by covering of the surface of the aeration tank, - consequently causing deficiency of the oxygen uptake by the mixed liquor. As bulking phenomena, filamentous foaming can lead to drastic operational problems in the treatment process (Tandoi *et al.* 2017):

- Visual problems, such as slippery pathways covered by escaping foam;
- Floating biomass in the secondary clarifier, deteriorating the final effluent quality;
- Accumulation of significant amounts of biomass into the foam, not active in the treatment process, leading to loss of ability to control sludge age.

The most common foaming phenomenon is caused by excessive proliferation of free filaments in the aeration tank and in the secondary clarifier (Wang *et al.* 2016). These free filaments tend to float either in the aeration tank or in the secondary clarifier, at the air-water interface, favouring evaporation. Unlike foams caused by detergents or other surfactant substances, these foams lead to a true separation of the

phases. This may lead to confusion between this phenomenon and the filamentous bulking phenomenon, as sludge may also be on the surface of the aeration tank or the secondary clarifier (Tandoi *et al*. 2017). So, the two phenomena are very often regarded as similar and treated in the same way. The microscopic observation of filamentous foam shows a strong concentration of filamentous organisms in relation to what occurs in the mixed liquor. Table 2.12 summarizes the types of foam and the causes associated to its occurrence.

Table 2.12: Type of foams and their related causes (adapted from Khodabakhshi *et al*., 2015)

Type of foam	Causes
Stiff white	Very low mean cell residence time (MCRT), high F/M, pH < 6.5 and pH > 9
Heavy brown	Growing of Nocardioforms and filamentous bacteria, nitrification
Thick brown	Old sludge, high MCRT, low F/M, high MLSS, low pH

Initially, the formation of biological foam was linked to *Nocardia amarae* because it was the first species to be isolated, described and identified (Lechevalier & Lechevalier 1974). However, more recent surveys have revealed that other filamentous bacteria can cause sludge foaming, such as *Microthrix parvicella* (Wang *et al.* 2016), other Nocardioforms (Chen *et al.* 2010) and several Eikelboom morphological types (e.g. Type 021N, Type 0041 and Type 1863). Glymph (2005); Ovez & Orhon (2005) and Guo & Zhang (2012) reported over 30 cultured species of foaming bacteria.

2.3.1 Factors affecting filamentous overgrowth phenomena

In general, the excessive growth of the filamentous forms can be attributed to a restricted group of factors, based in wastewater (substrate) characteristics, process design parameters, and treatment plant operating conditions (Kämpfer 1997; Jenkins *et al.* 2004). In Figure 2.8 the factors influencing filamentous organism growth in activated-sludge systems are outlined.

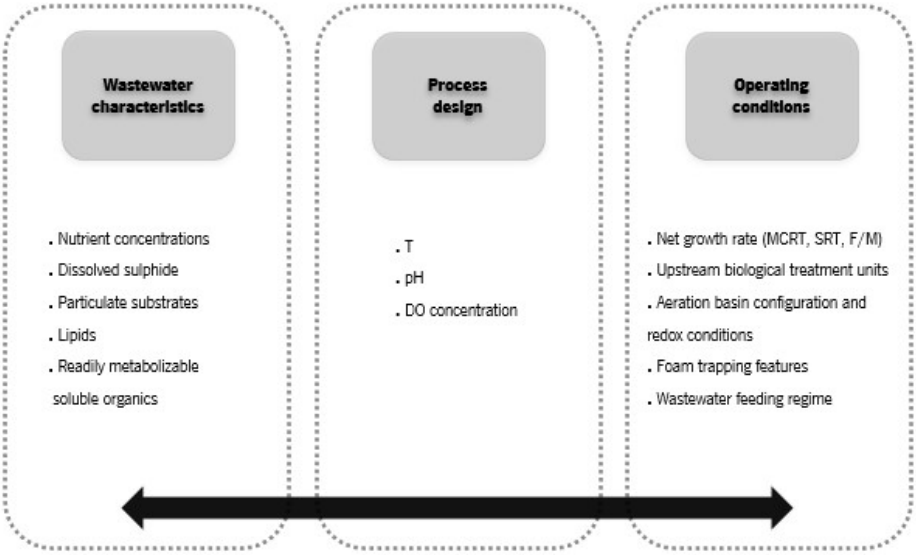


Figure 2.8: Factors of activated-sludge process favoring the occurrence of filamentous bacteria (adapted from Jenkins *et al.*, 2004).

Chemical composition of the influent wastewater and its provenience (domestic or industrial) and the nutrient balance seem to be crucial in the establishment of the filamentous community. Jenkins (1992) and Wanner (1994) suggested a classification of the filamentous bacteria based on their nutrient requirements (Table 2.13).

Table 2.13: Classification of filamentous bacteria on the basis of their nutrient requirements (adapted from Kämpfer, 1997 and Seviour & Nielsen, 2010)

Group of bacteria	Properties
S. natans, Type 1701 and Type 0041/0675	Strictly aerobic respiratory metabolism. Can store polyphosphate and poly-β-hydroxybutyric (only incidentally). Found in influent with high organic compounds, low DO levels and short retention time. Do not often occur in anaerobic/aerobic selector plants
Thiothrix spp. and Type 021N	Aerobic organisms with strictly respiratory metabolism. Do not often occur in anaerobic/aerobic selector plants but some can oxidize sulphur
Candidatus M. parvicella, Type 0092, *N. limicola* and Type 0803	Facultatively aerobic chemoorganoheterotrophs able to perform aerobic and anaerobic respiration. Frequent in plants with anoxic or anaerobic zones.
Mycolata like, *Gordonia* spp., *N. limicola*, Type 0092, Type 0041/0675 and *Acinetobacter* spp.	Hydrophobic cells, forming and stabilize foams. Occur often in presence of surfactants, grease or oil

The various filamentous microorganisms differ in terms of substrate and nutrient requirements, metabolic capacity, and resistance to several environmental factors (Seviour & Nielsen 2010). Therefore, the identification of the causes of unbalanced filamentous growth also calls for the knowledge of the ecological

characteristics of each species or type. Table 2.14 displays some of these microorganisms and the causes associated with their grow in activated-sludge (according to Jenkins *et al.*, 2004).

Table 2.14: Causes related with filamentous growth in activated-sludge (adapted from Jenkins *et al.*, 2004 and Seviour & Nielsen, 2010)

Cause	Filamentous bacteria
Low levels of dissolved oxygen	*S. natans*, *H. hydrossis* and Type 1701
Low F/M. Longer sludge ages or higher MCRT	*Candidatus M. parvicella*, *H. hydrossis*, *Nocardia* spp., Type 021N, 0041/0675, 0092, 0581, 0961, 0803 and 0914
High F/M, short sludge age (< 5 days)	Type 1863
Nutrients (N, P) deficiency	*Thiothrix* spp., *S. natans*, *H. hydrossis*, Type 021N and 0041/0675
Readily biodegradable soluble substrates	*S. natans*, *Thiothrix* spp., *H. hydrossis*, *N. limicola* and Type 1851
Gradually biodegradable or particulate substrates	*Candidatus M. parvicella,* Type 0041/0675 and 0092
Low pH (< 6)	Fungi
Presence of H_2S and septic wastewater	*Thiothrix* spp., *Beggiatoa* and Type 021N

Hydrolysis of macromolecules and high ability to remove organic matter seems to be an added-value of filamentous presence in activated-sludge, reinforcing that the type of organic matter plays a role in filamentous community composition (Mielczarek *et al.* 2012). Jones & Schuler (2010) suggested that in full-scale WWTP the seasonal variations in biomass density, settleability, besides filament content, play a role in seasonally variable settleability in the secondary clarifier. These evidences helped to clarify previously unexplained reports of seasonally variable settleability that were independent of variations in filament content and provided the basis for developing strategies to improve performance in these WWTP.

Avella *et al.* (2011) adopted an interesting approach combining information retrieved from the WWTP databases by statistical methods with the sludge physico-chemical parameters from three paper-mill treating systems. They concluded that this combined approach allowed a better understanding of the whole system, contributing to a good sedimentation of the sludge. In the same year, Muela *et al.* (2011) did not find any relation between physico-chemical and microbiological parameters in a Spanish WWTP. Their results showed that microbiological parameters were more sensitive than the physico-chemical to changes in WWTP condition, suggesting that microbiological parameters are essential to monitor the WWTP operation.

As evidenced above, significant effort has been directed to understanding how and why some filamentous bacteria proliferate sufficiently to cause bulking and foaming phenomena, in the hope of finding a general explanation for this problem. The main causes of filamentous overgrowth are not yet fully understood and

keep on receiving considerable attention in the search for more efficient control strategies (Adonadaga & Martienssen 2016; Jiang *et al*. 2016). In part, this is due to the fact that the problem has generally been approached from either an engineering (general solution) or a microbiological (species specific) point of view. An integration of both approaches can help to have a clearer insight on the factors that promote the filamentous bacteria growth (Seviour & Nielsen 2010).

The microbiological approach focusses on the predominance of filamentous bacteria responsible for the bulking and foaming occurrences. While in the engineering approach, engineers have dealt with the operational problems of bulking and foaming differently, trying to come up with unifying theories to cover all filaments, without worrying too much about their precise identity in plants (Seviour & Nielsen 2010). The engineering approach was reviewed more fully by Martins *et al*. (2004), presenting a basic theoretical framework to understand the filamentous overgrowth. One of the hypotheses of the cause of filamentous overgrowth is the diffusion-based selection: according to this theory, substrate and nutrient gradient in activated-sludge flocs trigger filamentous growth, i.e. a large concentration gradient within floc favours selection of filamentous bacteria over floc-forming bacteria (non-filamentous) (Liu & Liu 2006; Figueroa *et al*. 2015). Kinetic selection theory is another hypothesis proposed by Martins *et al*. (2004), initially reported by Chudoba *et al*. (1973): authors stated that low soluble substrate concentrations provide competitive advantages to filamentous bacteria, leading to bulking sludge. However, recently, an alternative hypothesis suggests that it is not the substrate concentration as such but the micro-gradients of substrate concentration inside flocs that plays a more important role in the competition between filamentous and non-filamentous bacteria (Martins *et al*. 2011). Storage selection theory was also presented by Martins *et al*. (2004). Storage is an intrinsic property of microorganisms, and traditionally, non-filamentous microorganisms are supposed to exhibit the ability to store substrate under high substrate concentrations (Tampus *et al*. 2004). However, recent studies showed that bulking sludge can have similar or even higher storage capacity than non-filamentous bacteria (Martins *et al.* 2004; Figueroa *et al.* 2015). This ability could be especially advantageous under the feast-famine regimes. The last theory proposed by Martins *et al*. (2004) is the nitric oxide (NO) hypothesis in which filamentous and non-filamentous bacteria are assumed to compete for organic substrate under different denitrification mechanisms. According to this hypothesis, nitrite and NO, both intermediates of denitrification, accumulate in the floc-forming bacteria and not in the filamentous bacteria. It was postulated that filamentous bacteria only perform denitrification till nitrite and, therefore, do not accumulate the intermediate inhibiting compound NO. In these conditions, filamentous bacteria have competitive advantages over floc-forming bacteria under aerobic period, because the intermediate inhibiting

compound NO will inhibit floc-forming bacteria proliferation under aerobic conditions (Martins 2004; Sheng 2015). However, these approaches do not entirely explain the phenomenon of filamentous bulking, and filamentous sludge bulking remains a serious problem in WWTP worldwide.

2.3.2 Controlling filamentous overgrowth phenomena

Understanding what affects proliferation of filamentous bacteria *in situ* might provide clues on how to control their overgrowth. Numerous studies have suggested and developed methods to control the filamentous organisms commonly identified in WWTP. The balance of filamentous to floc-forming bacterial populations, as was mentioned above, can be affected by physical-chemical and operational parameters. By appropriate modification of the influent, plant configuration and/or operation, the relevant causes may be removed, resulting in a decline of the filament content.

Filamentous bulking control strategies are usually divided into two types: specific and non-specific methods. Specific methods are considered preventive methods and aim at removing the cause of filamentous proliferation (targeted to a specific microorganism or group of microorganisms) and promoting the growth of floc-forming bacteria. So the specific methods include procedures that modify the conditions to create a hostile environment for the targeted filamentous organisms (Henriet *et al.* 2017) or to promote the growth of floc-forming bacteria. Therefore, the challenge is to find the appropriate environmental conditions to achieve this goal in such a complex environment as the aerated tank of the WWTP is (Tandoi *et al.* 2017).

Martins *et al.* (2004) stated that preventive actions for bulking sludge must not be based on the knowledge of the physiology and/or kinetics of a specific type of filamentous bacteria, despite the great emphasis in process monitoring on recognising the filamentous bacteria present in bulking. Several engineering solutions have been designed to lead to modifications of the microbial composition of the biomass in order to disfavour filamentous bacterial growth. It is clear that readily biodegradable substrates need to be consumed at high substrate concentrations, to favour floc-forming bacteria and disfavour the filamentous. In order to solve the problem without affecting substrate removal in the plant, the most useful strategy is to create a substrate concentration gradient inside the aeration tank. This goal can be achieved by modifying the activated-sludge process configuration from the completely mixed condition to one of three methods: plug flow reactors, introduction of selector reactors and sequencing batch reactors (SBR). In all, the purpose of the process configuration is to achieve the removal of most of the substrate when it

is present in high concentrations, where floc-forming bacteria are kinetically favoured, and to remove only a small fraction of the substrate when present in low concentrations, in conditions that favour filament growth. Sludge settleability in activated-sludge systems is then increased as filamentous growth decreases (Ferreira *et al.* 2014; Tandoi *et al.* 2017).

Additional studies were conducted to find other solutions to control bulking and foaming. Guo *et al.* (2010, 2013) developed a "low energy" system by reducing energy requirements for aeration and expected a limited or slight bulking sludge instead of eliminating bulking sludge. By using this strategy, a significant aeration energy was saved. Likewise, Tian *et al.* (2011) investigated the integrated N, P and COD removal performance under limited filamentous bulking using a bench-scale plug-flow enhanced biological P removal reactor (EBPR) fed with raw domestic wastewater. The results indicated the feasibility of limited filamentous bulking under low DO as a stimulation of simultaneous nitrification/denitrification for enhancing nutrient removal and satisfactory effluent quality in an EBPR process.

Another important approach to control bulking and foaming is the identification of the filamentous bacteria ecophysiological needs (Nielsen *et al.* 2009). By studying and understanding the ecophysiology of the filamentous bacteria (either in pure culture or by applying *in situ* techniques, such as microautoradiography or FISH to follow their densities), it is expected to find a solution to avoid the occurrence of the specific filament. The knowledge of the ecophysiology of microorganisms is crucial to develop a proper strategy for eliminating the particular group of filamentous bacteria (Miłobędzka & Muszyński 2016). Although most of these organisms are uncultured, and consequently the understanding of their physiology and ecology is still unsatisfactory, with the development of culture-independent molecular methods, knowledge of the identification of filaments and other populations in activated-sludge has increased dramatically, as well as better management of these processes.

Within the specific methods of sludge bulking control, a new and promising emerging field is being developed, relating the use of phages (viruses) specific for target filamentous bacteria. In 2011, Kotay *et al.,* applied a lytic bacteriophage (virus) to reach a mediated biocontrol of biomass bulking in the activated-sludge process using *H. hydrossis* as a model filamentous bacterium. Following the phage application, successful reduction in SVI was achieved. The application of this phage did not affect nutrient removal efficiency of the biomass, suggesting no collateral damage.

The non-specific methods aim at reducing filament levels without any control on the causes of their growth. Therefore, they are almost independent from filament type. Non-specific methods have only a

temporary effect (Henze *et al.* 2008); they are useful when the cause of filamentous bulking cannot be determined immediately or when a rapid resolution of the bulking problem is needed (Henriet *et al.* 2017).

Addition of chemical agents is one of the simplest approaches to bulking and foaming control and has been traditionally applied to contain these menaces. Chlorination was one of the first methods used to control filamentous bulking and is still used, though these substances also tend to damage floc-forming bacteria and to cause process breakdown (Lakay *et al.* 1988; Caravelli *et al.* 2003, 2006), so the relative survival between both should be evaluated (Guo *et al.* 2012). The correct choice of addition point and the determination of optimum dosage becomes essential to minimise the impact on the overall performance of the activated-sludge process. The purpose is to suitably control filamentous bacteria while minimising the effect on the floc-forming ones. Partial success and cases of failure following the employment of chlorine can be found in the literature (Fontaine 1999; Madoni *et al.* 2000). This action is often used as a short-term (transient effect) and cost-effective solution with the ultimate goal of dealing with a critical situation until a specific control measure can be implemented (Seka *et al.* 2001; Xu *et al.* 2014; Tandoi *et al.* 2017). Some authors claim that a sporadic chlorination can also be a permanent solution for cases where not-specific control measures have been developed yet or where it proves to be economical. Even though chlorination seems to be very effective in certain cases, its application in Europe is limited because of the potential formation of undesirable by-compounds, such as trihalomethanes, which are potentially dangerous to human health and ecosystems (Banach *et al.* 2015).The chlorination process may induce variations in effluent toxicological responses due to the presence of undesirable by-compound, and because nitrification process, phosphate and COD biological removal are also significantly affected using non-selective agents (Pal *et al.* 2014).

Surfactant addition constitutes an interesting alternative for the control of filamentous bulking, due to their ability to lysate filamentous bacteria. One of the advantages of the use of surfactants is their low cost and when properly used can be useful to solve bulking problems without affecting floc-forming bacteria (Caravelli *et al.* 2007). Petrovski *et al.* (2011) studied the role of surfactants in stable foaming incidents, and their influence on *Mycolata* foaming behaviour.

Polymer addition also proved to be very effective for the control of filamentous bacteria: Martins *et al.* (2011) studied the effect of a polymeric substrate (starch) on activated-sludge settleability. For this study, a polymeric substrate (starch) and an easily biodegradable soluble monomer (glucose) were used. The experiments showed that conditions leading to bulking sludge with glucose did not lead to bulking when starch was used. These results are in accordance with the kinetic selection theory, previously mentioned.

It was observed by the authors that in pseudo-steady state systems, starch hydrolysis takes place inside the flocs, not giving rise to strong gradients of substrate concentration over the floc radius. The concentration of monomers at floc level is, however, low and rate limiting, a condition that, according to the kinetic selection theory, would lead to bulking sludge. Nevertheless, micro-gradients of substrate concentration, as hypothesized by the diffusion-based theory, seem to be more important for the filamentous bacteria proliferation. In 1997, Shao *et al.* (1997) had already observed that small amounts of a cationic polyacrylamide polymer added to the aeration tank eliminated Nocardioform foam.

De Gregorio *et al.* (2011) evaluated the effect of the coagulant ferric chloride in the settling properties, the abundance of filaments and the microfauna populations in a laboratory-scale activated-sludge reactor fed with a synthetic wastewater of a dairy industry. The results showed that with a controlled coagulant dosage, an improvement in reactor performance was observed, if considering sludge settling properties and mainly with respect to the COD removal.

Filamentous bulking may also be controlled by adding flocculating agents. Its purpose is to overcome the bridging or diffuse floc structure associated with excessive filamentous organism growth (Seka *et al.* 2001). Their addition to the sludge resulted in the formation of larger and firmer flocs and yielded immediate sedimentation improvement. Mamais *et al.* (2011) studied the use of flocculant agents to increase the floc density and improve sludge settling ability.

In summary, non-specific control methods often proved to be unsatisfactory when the bulking episodes are recurrent due to their transient effect on the activated-sludge ecosystem. Furthermore, they are potentially detrimental for all the biomass (Rossetti *et al.* 2005). For this, they should be restraint to critical occurrences and never as a regular method to overcome bulking problems. On the hand, the specific control methods should be developed and preferentially be adopted (Henriet *et al.* 2017); their application would allow a permanent control of bulking in activated-sludge systems in a sustainable way.

2.4 Biocides

Biocides have attracted the attention of wastewater treatment technicians and researchers for the potential of its use in the control of filamentous overgrowth becoming one of the most commonly used methods to control filamentous bulking under acute conditions (Jenkins *et al.* 2004) and influence in the overall performance of activated-sludge processes. Furthermore, biocides increasing use within domestic,

industry and healthcare environments for disinfection purposes has raised concerns because of the potential effects of their dissemination to human health and to natural environments (Batt *et al.* 2017).

Biocides are non-antibiotic chemical compounds normally, constituted by inorganic or synthetic organic molecules, with disinfectant and antiseptic properties (sanitizers), as they can inhibit microbial growth or even kill microorganisms (bacteria, fungi and algae) when used correctly (de Carvalho 2007). These antimicrobials can also be used to preserve materials or processes from microbiological degradation (Bodík *et al.* 2008). Depending on the final expression of the specific biocide, it is possible to describe the nature of the biocide antimicrobial activity that it intends to ensure. For instance, the expression ending in the suffix "-static", such as "bacteriostatic", are used for agents that inhibit microbial growth without killing the microbes, and the expression with the suffix "-cidal", such as "fungicidal" and "bactericidal" refer to agents that kill the target microbe. The misuse of these substances in the diverse healthcare, industry and household cleaning processes might play a role similar to antibiotics in many ways (Levy 2001), as they might contribute to the resistance problem developed by bacteria (Smith *et al.* 2007; Ortega Morente *et al.* 2013; Bengtsson-Palme & Larsson 2016). The most significant industrial requirements of a biocide are that it must be effective against microorganisms, economical and compatible with the environment where it is applied (Ashraf *et al.* 2014).

2.4.1 Biocide oxidizing and non-oxidizing types

In general, biocides can be divided either in oxidizing or non-oxidizing, depending essentially on the mechanism used to kill target organisms.

Oxidizing biocides inhibit microbial growth and kill quickly microorganisms, being widely used in the water treatment plants and supply networks, electric power and refining industries because of their effectiveness, moderate cost and easy treatability. Some of the most widely used oxidizing biocides are chlorine and bromine, chlorine dioxide or ozone compounds, that have fewer damaging effects to the environment and human health than chlorine. When biocides, such as chlorine or bromine compounds, are added to water they form hypochlorous acid (HOCl) or hypobromous acid (HOBr), which act as the active ingredients (Russell 2003). The active ingredients in antiseptics and disinfectants achieve their effects through interactions with the microorganism cell surface followed by penetration into the cytoplasm and action on cellular targets. Historically, chlorine gas, as an oxidizing biocide, was widely used because of its low cost. However, in recent years, many users have switched to other forms of

chlorine because of the health and safety risks associated with its handling (Ashraf *et al.* 2014). Bleach (sodium hypochlorite) is being now widely used as a source of HOCl. Sources of HOBr are becoming increasingly popular in place of or in addition to sources of HOCl. The sodium bromide reacts with the HOCl to form HOBr, which is an effective microbiocide over a wider pH range than is HOCl. Other powerful oxidizing biocides that do not rely on chlorine or bromine as an active agent include ozone and hydrogen peroxide, although these two methods have higher costs compared to chlorination.

The non-oxidizing biocides, though cover a wider spectrum of chemical types, are generally stable, have longer lasting effects and therefore slower kill features. Some of the limitations of non-oxidizing biocides may include concerns for environmental toxicity, reactivity with additives (e.g., cationic biocides) and higher costs (Amjad 2010). These biocides typically interact with the metabolism of the cell (altering its permeability) or affect its structural integrity (Kelland 2014). The most common examples are aldehydes, as glutaraldehyde, formaldehyde, and ortho-phthalaldehyde, isothiazoline, organo-bromide, organo-tin/quaternary ammonium salts, quaternary ammonium salts, triazine, dodecylguanidine hydrochloride (DGH), carbamates, methylene bis-thiocyanate (MBT), and dibromonitrilopropionamide (DBNPA). Quaternary ammonium salts (Quats) are one of the products widely used in electric power industry. There are numerous non-oxidizing chemicals used as primary biocides or as supplements to oxidizing biocide applications. Frequently, a combination of oxidizing biocides and non-oxidizing biocides is used to optimize the microbiological control (Mohan & Srivastava 2010).

2.4.2 Biocide mode of action

Understanding the mode of action of biocides is important for optimizing their rational use and combating resistance if encountered (Williams 2007). Indeed, the increased use of biocides at different ranges of concentrations has led to significant scientific debate regarding their role in bacterial survival and resistance. Therefore, nowadays, it is still necessary to investigate further the nature of the inhibitory and lethal effects of biocidal disinfectants on a range of microorganism's entities. Concerns have been raised in recent years regarding that in certain circumstances bacteria may become unsusceptible to some biocide regimes, or that a reduced susceptibility to biocides may promote an elevated degree or frequency of antimicrobial resistance. The rise of antimicrobial resistant populations has become a concern on a global scale (Khan *et al.* 2017; Wales & Davies 2015).

The array of possible cell multi-target sites forms an important aspect of the studies tackling biocides mode of action, efficacy and relevance to disinfection purposes. Table 2.15 shows the characteristics on which the activity of the biocides against microorganisms depends. The concentration of the antimicrobial agent, the temperature or pH at which it is acting, can have profound influence on its activity. However other parameters must also be considered (Braga 2011; Fraise *et al.* 2013)

Table 2.15: Characteristics on which the activity of biocides against microorganisms depends (adapted from Falkiewicz-Dulik *et al.,* 2015)

Characteristics
External physical environment
Nature, structure, composition and condition of the organism itself
Ability of organism to degrade or inactivate the particular compound converting it to an inactive form

This type of chemical agents has a diversity of modes of action such as the attack of the cell wall (cell viability), of the cytoplasm or the cellular membrane, depending on chemical properties, microorganisms and environment. The interaction between a biocide and a microoganism follows a sequence of actions in which the biocide, in the first place, binds to the microbial cell surface, and then penetratres the cell wall and membrane, entering the cytoplasm, where it can interact with cellular proteins or nucleic acids (Braga 2011). The toxic action results from the cellular damage caused by the biocide at some phase during this process (Fraise *et al.* 2013). Antimicrobial substances can also target the respiratory functions, the enzymes and genetic material (Bodík *et al.* 2008). Biocides have also the ability to affect the initial adhesion of microorganisms in biofilms establishing by changing the surface free energy of the substratum or the bacterial wall hydrophobicity (Pereira & Vieira 2001). Potential targets are the cell wall or outer membrane, the cytoplasmic membrane, the functional and structural proteins, the DNA, the Ribonucleic acid (RNA) and other cytosolic components (Bridier *et al.* 2011). Consequently, susceptibility to biocides varies between Gram positive and Gram negative bacteria, being the Gram negative bacteria reported to be less susceptible to biocides. This difference is thought to be due to the fact that the Gram negative bacteria may have a high cell impermeability, so that the access of the biocide to its target sites can be reduced, causing the cells intrinsically less susceptible to biocides (Johnson *et al.* 2002; Falkiewicz-Dulik *et al.* 2015). A summary of the different sites of interaction between biocides and microorganisms are provided in Figure 2.9.

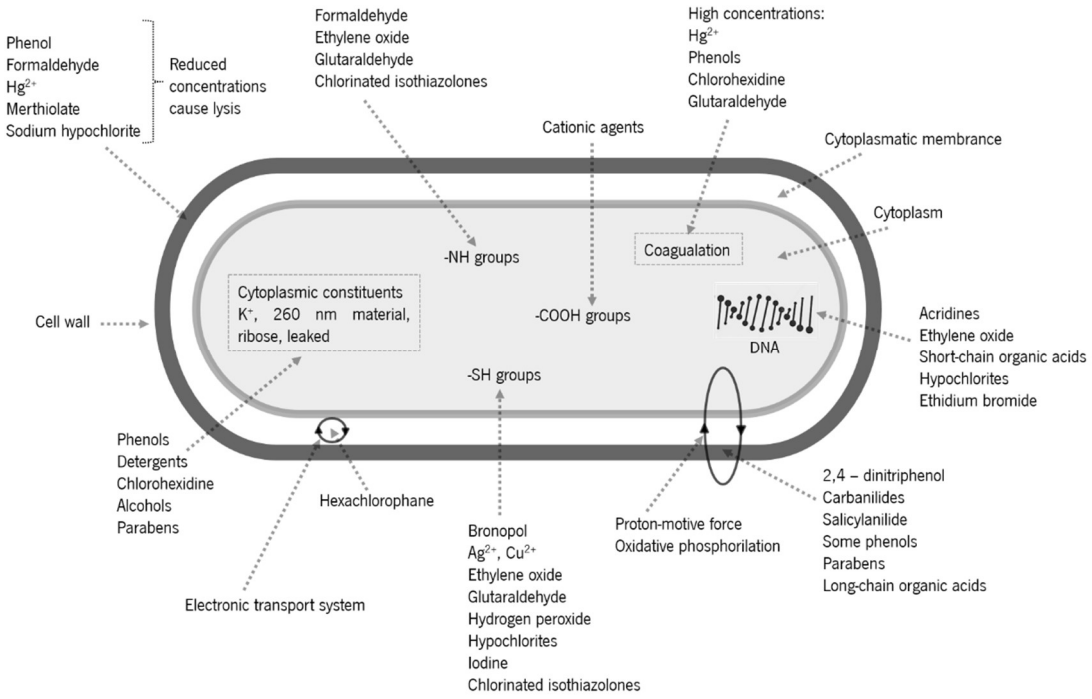


Figure 2.9: Sites of action of biocides (adapted from Fraise *et al.*, 2013).

De Carvalho (2007) reported that a biocide should have a wide range of activity, both in terms of the type of susceptible microorganisms and the conditions of action, and should not be easily inactivated. It is also intended that a biocide should be able to prevent biofilms from forming, since the formation of biofilms and the consequent decrease of the susceptibility to the biocides, has important implication in the clinical settings, contributing to contamination recalcitrance and spread of infectious microorganisms between patients, as well as in several sectors, leading to the contamination of the product (Braga 2011). Biofilms are self-organized communities of sessile microorganism adhered to a surface and encased in a matrix of extracellular polymeric substances (EPS) that they produce (Cydzik-Kwiatkowska & Zielińska 2016). In biofilm control through the use of a biocide, the microbial response to the agent will depend not only on the type of microorganisms encased within the biofilm and the type of chemical agent, but also on the complex interactions between the biocide and the EPS biofilm matrix. In fact, the use of such chemical agents might cause changes to the EPS matrix (where bacterial colonies protected from antimicrobial agents are found) that can affect the biofilm structure (Pereira & Vieira 2001; Chen *et al.* 2017).

Biocide mechanisms of action over various microorganisms can be divided into four general broad categories (Figure 2.10), organized based on the target region of the microorganism affected by biocide action. As previously stated, biocides will act on microrganisms in an outside to inside direction, which means that the cell wall, cell membrane/membrane-associated components, and cytoplasmic regions

will be successively affected by a biocide as it interacts with the intended target organism. The chemical struture of a biocide determines its afinity for specific targets and is the key to understanding its mode of action (Lederberg 2000).

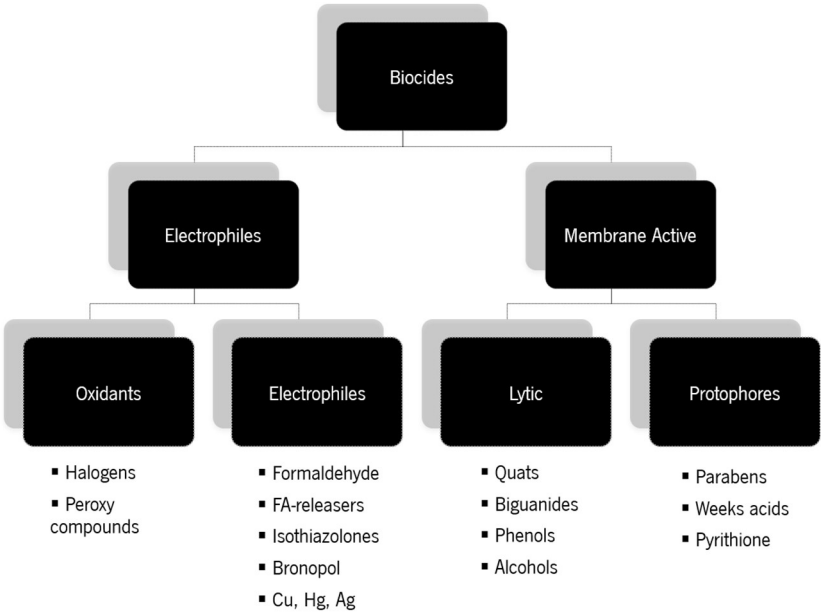


Figure 2.10: Mechanisms of biocides action (adapted from Falkiewicz-Dulik *et al.*, 2015).

The biocides functioning as oxidants include agents with rapid speed of kill, such as chlorine and peroxides, that diffuse toward the surface of the microorganism and then permeate into the membrane and cytoplasm, oxidizing organic material (Chapman 2003; Falkiewicz-Dulik *et al.* 2015). Inactivation or loss of viability occur when vital constituents suffer a certain level of irreversible damage (Caravelli *et al.* 2006). These last authors reported that chlorine diffuses through the cell walls, produces a dysfunction in internal enzymes and hence inactivates cells. Regarding ozone, its disinfection action is a direct result of cell wall disintegration and cell lysis. The electrophilic agents include inorganic ions such as silver, copper, and mercury, and organic biocides such as formaldehyde and Isothiazolones (Chapman 2003; Falkiewicz-Dulik *et al.* 2015). These biocides react with critical enzymes to inhibit growth and metabolism, and cell death occurs after several hours of contact (Fraise *et al.* 2013). Cationic membrane active biocides, such as quaternary ammonium compounds (QACs), phenols and alcohols tend to directly affect cell membranes (cell lysis) (Williams 2007; Kelland 2014). Seka *et al.* (2001) stated that the most probable mode of action of QACs as biocides reported in literature is their effect on the plasma membrane. Protonophore, such as parabens, weak acids and pyrithione, interfere with the ability of the cell

membrane to maintain a proper pH equilibrium, which leads to acidification of the cell interior with extensive disruption of metabolism (Chapman 2003; Falkiewicz-Dulik *et al.* 2015).

2.4.3 Environmental impact

Despite the wide use and the probable exposure of humans to biocides, scarce information is available about exposure concentrations and no systematic measurements are provided, or remain limited, for the home environment (Hahn *et al.* 2010; Dodson *et al.* 2015; Benson *et al.* 2017). Recent studies have revealed biocide residues in a wide range of ecosystems and organisms, playing an important role in introducing non-antibiotic-related contamination into the environment (Arnold *et al.* 2014). It is known that the same antimicrobial may be used in varied species and applications, being the biocidal strategy designed to have biological effects at both high and low doses. This use of biocides at different ranges of concentrations in healthcare, household and industry often may impact the environment as small amounts of biocide can be released progressively contributing to the wide-range of concentrations of biocidal compounds that normally are found in the environment. Nevertheless, those concentrations are at significantly lower orders of magnitude as compared with those used in disinfection settings (Franklin *et al.* 2016).

Overall, there is a demonstrable lack of knowledge and agreement about the frequency and extent of occurrence, fate, and effects associated with the antimicrobials entering the environment (Arnold *et al.* 2014). Indeed, the environmental impacts are not completely understood so far. The determination of the risk of antimicrobials in the environment may be dependent on the respective biodegradability and adsorption in relation to the concentration, stability, and persistence of a drug in the ecosystems, as well as the effects of T, available oxygen, pH and other environmental factors (Walsh 2013). There are some differences among biocides with regard to their major breakdown products. The breakdown products of biocides and their qualitative effect on the environment are shown in Table 2.16.

Table 2.16: Environmental fate of biocides (adapted from Doyle *et al.*, 2006)

Active biocide	Breakdown products	Environmental impact
Chlorine dioxide	Chloride and chlorate ions, or chlorite and chlorate	Minimal to high
Peracetic acid	Acetic acid, water and oxygen	Minimal to none
Peroxyoctonoic	Octanoic acid, water and oxygen	Minimal to none
Iodophor	Surfactant and iodine salt	Depends on surfactant
Acidified sodium chlorite	95 % chlorine (Cl)	None to high dilution
QACs	Mineralizations – readily to ultimately biodegradable	Might affect waste treatment plants at high concentration

2.4.4 Biocides in WWTP

As aforementioned, the extensive use of biocides in many industries and disinfectants in house and medical settings to control growth of planktonic and/or sessile microorganisms (in a biofilm) can have environmental and ecological issues, since sooner or later biocides residues can be released into wastewater and reach the aquatic environment. So, knowledge of the effects of biocides on wastewater treatment units and their effectiveness against bacterial cells of the WWTP is of upmost importance (Laopaiboon *et al.* 2006).

WWTP are increasingly becoming a target for regulatory and public pressure with regard to their discharges to the environment (Stamatis *et al.* 2010; Naidoo & Olaniran 2014). Using substances that negatively influence microorganisms, by entailing a biological imbalance, can lead to an incorrect WWTP operation (Bodík *et al.* 2008; Surerus *et al.* 2014). The transport of biocides residues through urban drainage systems and WWTP to the receiving waters, is an important pathway for contamination of the aquatic environment. Due to the increased resistance of microorganisms to chemical antimicrobials, the concentrations need to successful disinfection are raising, leading to the augmentation of disinfectant residues on the wastewater systems that can be highly toxic, requiring stronger environmental restrictions when discarded in water resources. Also, other indirect effects cannot be excluded, such effects on soil microbiota and ecosystems that are determined by the antibiotic's presence.

Levy (2001) and Roca *et al.* (2015) clearly suggest that antibacterial agents will have an impact on the environmental flora and on resistance emergence. In fact, resistance will certainly increase as the drug persists in the natural environment, especially at low levels for long periods of time representing a risk for the living species of the ecosystems. With increased concern regarding the potential adverse effects of biocides in aquatic environments even to humans, the removal from WWTP of commonly used biocides

is increasingly important (Liu *et al.* 2017). Besides the possibility of directly affect the natural receiving waters, biocide residues can also interfere with the environmental microbial flora of WWTP by affecting the susceptible bacterial strains, namely filamentous, and selecting bacteria resistant to them. Nowadays, with the tight restrictions regarding the disposal of biocides in WWTP, some strategies have been implemented, including the use of various alternatives to the conventional substances, the implementation of judicious or prudent biocide use guidelines and the implementation of national resistance monitoring programs (Lee *et al.* 2013). Research on the fate of biocides along wastewater treatment processes is scarce and few studies are available (Petrie *et al.* 2015).

On the other hand, surfactants, namely CTAB, are frequently used to improve the dewaterability of sludge (Guan *et al.* 2017), enhancing the overall treatment. Surfactants improve the disintegration of sludge flocs by promoting the dissolution of EPS (*Shi et al,* 2021).

In this project, three biocides: a phenolic compound (triclosan), a quaternary ammonium compound (CTAB) and an aldehyde-based biocide (glutaraldehyde) were tested, with the main purpose of gaining insights into the impact of these chemicals in the biological communities of WWTP, as potential control compounds of the filamentous bacteria.

2.4.5 Triclosan

Triclosan, an oxidizing biocide belonging to the phenolic compound biocide group was tested in this work. Triclosan is a widely used broad-spectrum bactericide, due to its effectiveness and thermal stability, found in a wide range of consumer products (Carey & McNamara 2015). It can be found more specifically in personal care products, such as toothpaste, antibacterial soaps, dishwashing liquids, cosmetic and antiseptic products and antiperspirants/deodorants (Dhillon *et al.* 2015). The action mechanism of triclosan focuses on the induction of cellular damage, causing cellular contents to leave (physically) the cell membrane, when in the presence of bactericidal (higher) concentrations. At low concentrations (bacteriostatic), it can effectively prevent bacterial lipid biosynthesis by blocking enzyme enoyl-acyl carrier protein reductase (ENR) (target site for the action) (Lee *et al.* 2013; Dhillon *et al.* 2015). It is reported that triclosan has a broad-spectrum idem activity against Gram positive and Gram negative bacteria (non-sporulating bacteria), as well as against some yeasts and fungi (Fraise *et al.* 2013; Johnson *et al.* 2002). At low concentrations, triclosan inhibits the growth of microorganisms, whereas at higher concentrations the effect is deadly to the organisms (Dhillon *et al.* 2015). Triclosan is fairly insoluble in aqueous solutions,

unless the pH is alkaline, and readily soluble in most organic solvents (Schweizer 2001) acting as a site-directed, picomolar inhibitor of ENR by mimicking its natural substrate. Triclosan, after use, is considered as a residue, which when not biodegraded during the wastewater treatment, may enter the aquatic environment through treated wastewater effluent or sludges (Capdevielle *et al.* 2008). Lee *et al.* (2013) described that biodegradation is an important removal mechanism for triclosan in the WWTP, and some microorganism presents in wastewater, such as *Sphingomonas* sp. Rd1, *Nitrosomonas europaea*, *Sphingomonas* sp. PH-07 and *Sphingopyxis* strain KCY1, are known to degrade triclosan via cometabolic reactions. It was also described those two soil bacteria are known to use triclosan as the single source of carbon, but it remains unknown whether any wastewater microorganisms can use triclosan as a carbon source. Carey & McNamara (2015) stated that triclosan has been associated to the alteration of the structure or function of the microbial community in the WWTP, namely in activated-sludge and anaerobic digestion. It is further reported that the concentrations of triclosan found in these communities are not those used for growth inhibition, and the concentrations normally encountered in the environmental (sub-μg/L concentrations) may cause selective pressure in bacteria, which subsequently respond to develop resistance mechanisms, leading to inhibition of the microbial community. Schweizer (2001) pointed out that there is a link between triclosan and antibiotics, and the widespread use of triclosan-containing antiseptics and disinfectants, causing the increase of triclosan concentration in the aquatic and terrestrial environment (Dhillon *et al.* 2015), which may indeed aid in the development of microbial resistance, in particular cross-resistance to antibiotics. Concern about its impact on the resistance to antibiotics in the environment is increasing (Carey & McNamara 2015). More recently, another area of concern with triclosan is the production of chloroform. It is hypothesized that triclosan may increase the production of chloroform, a potential carcinogen (Lee *et al.* 2013; Dhillon *et al.* 2015). Because of the extensive use of this antimicrobial in personal hygiene products, humans are commonly exposed through ingestion (e.g. toothpaste) or dermal absorption (e.g. soaps) (Gautam *et al.* 2014).

2.4.6 CTAB

QACs are broadly used as topic antiseptics and may be present in many household products. In this work, it was used CTAB as a non-oxidizing and a cationic surfactant. The use of QACs for sludge bulking control has been attempted with success for a many years (Hwang & Tanaka 1998).The addition of chemical surfactants may be an effective method to control filamentous overgrowth due to their enhanced ability to induce filamentous cell lysis (Guo *et al.* 2012) by adsorption on cell wall/cytoplasmic membrane

(Ludensky 2005; Abreu *et al.* 2013). CTAB is an effective biocidal agent against bacteria and its action against filamentous bacteria was reported previously (settling problems) (Bitton 2005; Yang *et al.* 2013; Bratby 2016), with a more biocidal effect on the cell lysis of Gram negative filamentous bacteria, such as Type 021N and *Thiothrix* I, than on Gram positive filamentous bacteria (e.g. *Nostocoida limicola* II) (Seka *et al.* 2001). This can be explained by the presence of a hydrophobic cell wall in the Gram positive filamentous bacteria. More recently, Guo *et al.* (2012) described the effect of CTAB on filamentous bulking sludge caused by chlorine resistant bacteria Type 021N, reporting that CTAB, being a cationic compound, may have a stronger penetration capacity through the cell wall. The cationic surfactants have a ready attraction, to and absorption on, negatively charged surfaces such as bacteria (sewage sludge, soil and sediments) (Zhang *et al.* 2015), which allows a prolonged effect compared to chlorine (e.g.) (Seka *et al.* 2001). However, the application of CTAB for sludge bulking control is not yet widespread. There are two problems needing to be solved: CTAB higher operational cost (compared to chlorination) and the occurrence of foaming at high concentrations that may affect WWTP performance (similar phenomena had already been reported after the QACs additions) (Guo *et al.* 2012; Xu *et al.* 2014). Alternatives to reduce the problems associated with this are, for instance, the use of a synthetic polymer, creating a multi-component approach, capable of controlling the filamentous bulking (improves sludge settleability) and prevent the foaming phenomenon (Juang 2005; Tandoi *et al.* 2017). The adverse impacts of CTAB for the environment and receiving water must be taken into consideration. Continuous exposure to CTAB in different environments may result in the development of multiple resistance or co-resistance. However, the CTAB harmful effects to the environment are expected to be reduced or insignificant if properly added (Guo *et al.* 2012; Romero *et al.* 2017).

2.4.7 Glutaraldehyde

Glutaraldehyde (an aldehyde-based biocide) is also a non-oxidizing antimicrobial agent. Pereira & Vieira (2001) stated that glutaraldehyde is widely used in industry and hospital environments, since it has a broad range of activity (against bacteria and their spores) and high effectiveness, especially with difficult and persistent organisms due to its good penetrating ability. Glutaraldehyde also has a rapid rate of killing (minutes to hours). Since the introduction of the biocides, in 1945, glutaraldehyde (within the aldehydes), among others, has been the most important agent (Russell 2002; Braga 2011). From very early on that glutaraldehyde is used as fixative, as it kills the microorganism, but maintains its structure; for example, in Schuppler *et al.* (1998) glutaraldehyde is used as a fixation protocol (pre-treated) in cell wall

permeability, making them more permeable for penetration by the FISH probes. Glutaraldehyde biocidal activity involves the cross-linking of the outer proteinaceous layers of the cell in such a way that cellular permeability is altered (Laopaiboon *et al.* 2006). The action mechanism of glutaraldehyde is based on its two aldehyde end groups, these groups being chemically very reactive, capable to interact with constituents of the wall and cellular membrane of the microorganisms (Pereira 2001). In Fraise *et al.* (2013) it is reported that glutaraldehyde is more stable at acidic pH, but more potent in lethal effects in the presence of alkaline pH. The cross-linking mechanism of glutaraldehyde is also influenced by time, temperature and even concentration. Glutaraldehyde is often combined with other surface-active agents and biocides, such as quaternary ammonium or phosphonium compounds, to increase the speed with which it kills bacteria and reduce the dosage of glutaraldehyde (by itself would be quite high) (Kelland 2014). Further, Ludensky (2005) stated that glutaraldehyde displays particular strength in controlling sulphate-reducing bacteria. *Beggiatoa*, *Thiothrix*, and Type 021N are the filamentous sulphur bacteria present in activated-sludge systems, and the rapid and undesired growth of *Thiothrix* and Type 021N often occurs in activated-sludge processes, contributing to settleability problems and loss of solids from secondary clarifiers (Gerardi 2006). Glutaraldehyde is generally considered to be a good mycobactericidal agent (Fraise *et al.* 2013).

Due to the chemical diversity of the different contaminants arriving at the WWTP, no single methodology is capable of being a standard for analysis (Batt *et al.* 2017; Rizzo *et al.* 2019).

Seen as a promising direction of research to know how to take advantage of the biocides entry in sub-lethal concentrations in the WWTP, by studying to what extent they can affect the microbial community and whether its presence can be a measure of filamentous bulking control, is a problem still far from being solved.

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CHAPTER 3

Study of 16 Portuguese activated-sludge systems based on filamentous bacteria populations and their relationships with environmental parameters

In this chapter, the results of a survey of 16 Portuguese urban activated-sludge WWTP are analysed to improve the understanding of the environmental parameters determining the composition of the filamentous community in the aeration tank. A total of 128 samples of mixed-liquor from municipal WWTP were collected during two years and 22 filamentous morphotypes were identified. The most frequent and abundant filamentous bacteria were, in both cases and by this order, Type 0041/0675, Type 0092, *M. parvicella* and Type 1851, Nocardioforms and *H. hydrossis*. Concerning dominance, Type 1851 was the most frequently dominant morphotype, followed by *M. parvicella* and Types 0092 and 0041/0675. These were also, and by the same order, the dominant morphotypes during bulking occurrences. Significant correlations were obtained between the abundance of filamentous bacteria and environmental parameters, but multivariate statistical analysis only confirmed the negative correlation between Type 0092 and SVI, emphasizing the association of this particular filamentous organism with bulking. Discussion of the results considering published works was complicated by the random use of terms such as frequency, abundance, and dominance with different and often unclear meanings. This reinforces the need of clarifying these terms when discussing the causes of filamentous overgrowth in WWTP.

This chapter corresponds to the following publication: Araújo dos Santos, L., Ferreira, V., Neto, M.M., Pereira, M.O., Mota, M., Nicolau, A. (2015). Study of 16 Portuguese activated sludge systems based on filamentous bacteria populations and their relationships with environmental parameters. *Applied Microbiology and Biotechnology*, **99**(12), 5307–5316

3.1 Introduction

Activated-sludge process is the most common technology for treating several types of wastewaters, including domestic sewage and industrial effluents. The diversity of the biological community inhabiting the aeration tank of these plants is quite large, containing many species of viruses, bacteria, protozoa, fungi, little metazoa, and even algae. In this complex ecosystem, bacteria represent approximately 95 % of the total microbial biomass (Jenkins *et al.* 2004). Bacteria in activated-sludge can be divided into floc-forming and non-floc forming forms, being the latter mostly filamentous bacteria. It is assumed that bacteria that are kept in the activated-sludge process under the correct environmental conditions, that is, with sufficient oxygen and balanced food, can efficiently remove the organic material and nutrients from wastewater effluent (Martins *et al.* 2004). One must take in consideration, however, that a good separation (settling) and compaction (thickening) of activated-sludge in the secondary clarifier is a necessary condition to guarantee a good effluent quality (Martins *et al.* 2004).

As mentioned in the previous chapter, some problems associated with the activated-sludge technology - the development of a thick viscous scum or foam on the surface of activated-sludge aeration tanks and clarifiers (known as foaming) and the inadequate solid separation (or bulking) - are usually connected with an unbalanced growth of filamentous microorganisms. Serious problems of filamentous bulking and foaming are worldwide known and lead to poor treatment quality. Eikelboom (1975) developed the first identification key to identify filamentous bacteria in activated-sludge systems, mainly based on morphological characteristics and on the response of the filamentous bacteria to a few microscopic staining tests. Reference manuals were published with the description and characterization of 30 different filamentous bacteria commonly found in activated-sludge (Eikelboom 1975, 2000; Jenkins *et al.* 2004). Meanwhile, some publications discussed in detail the reasons for the excessive proliferation of filamentous bacteria (Eikelboom 2000; Jenkins *et al.* 2004; Nielsen *et al.* 2009; Seviour & Nielsen 2010; Tandoi *et al.* 2017).

Conventional microscopic sludge analysis is, and will be, an essential monitoring instrument tool for the technicians, as it provides information about sludge quality and operation conditions, besides detection of filamentous bacteria (Müller *et al.* 2007). On the other hand, the difficult and ambiguous identification of filamentous bacteria by conventional microscopic techniques led to molecular methods based on DNA or RNA analyses of the bacteria (Seviour & Nielsen 2010). FISH with DNA probes which employ fluorescently labelled rRNA-targeted oligonucleotides is used to confirm identifications, to locate specific organisms, and to give more detailed information on the various taxonomic levels.

The aim of the present work was to contribute to the knowledge of how environmental parameters (physical-chemical and operational) determine the composition of the filamentous community in activated-sludge. For that, a set of 16 activated-sludge plants were followed during two years and the relationships between the physical-chemical and operational parameters and the filamentous organisms were inspected through statistical analysis.

3.2 Experimental methods and procedures

3.2.1 Wastewater treatment systems

The study was performed in 16 urban activated-sludge WWTP, located in the north of Portugal (region of Minho) with nutrient removal. These WWTP are considered urban WWTP because they receive domestic and industrial sewage in different amounts, and even in the case of significant amounts of industrial residual water, this is not quantitatively or qualitatively defined and varies along time.

3.2.2 Sampling and preservation

Mixed-liquor samples were collected from the aeration tank every three months, during two years, in a total of 128 samples. The samples were sent by express mail and arrived at maximum 5 h after collection. Once in the laboratory, they were microscopically examined and stored at 4 °C for further confirmation if necessary. Gram and Neisser smears were also made.

Sub-samples were fixed prior to hybridization. They were preserved in paraformaldehyde and ethanol for the analysis of Gram negative cells and in ethanol for the examination of Gram positive cells, and then stored at -20 °C.

3.2.3 Physical-chemical and operational parameters

Physical-chemical variables were determined according to the *Standard Methods* (APHA 1998): pH, COD, BOD_5, total suspended solids (TSS), TP, TN and ammonium-nitrogen (NH_4–N) from affluent (sewage) and effluent. Nitrate-nitrogen (NO_3–N) was also measured in effluent. pH, SVI, SRT, F/M, DO and MLSS from aeration tank. Minimum, maximum, mean values, and standard deviation (SD) of each parameter during the period of study are shown in Table 3.1.

Table 3.1: Physical-chemical and operational parameters observed in the urban WWTP during the period of study

		Minimum	Maximum	Average	SD
Affluent	pH	6.18	9	7.42	0.50
	COD (mg/L)	12.8	4540	589.75	463.90
	BOD (mg/L)	22	1000	308.21	204.60
	TSS (mg/L)	30	1240	267.52	185.70
	TP (mg/L)	1	185	33.79	44.90
	TN (mg/L)	12	355	97.64	54.50
	NH_4-N (mg/L)	2	230	65.71	56.40
Aeration tank	pH	6.05	8.39	6.95	0.38
	SVI (mL/g)	36.23	403.23	212.15	82.86
	SRT (d)	0	334.1	25.79	39.74
	F/M (kg BOD_5/kg VSS.d)	0.01	0.77	0.09	0.10
	DO (mg/L)	0.17	5.19	1.41	0.76
	MLSS (mg/L)	1825	7815	3341.45	879.14
Effluent	pH	5.88	8.51	7.04	0.41
	COD (mg/L)	3	93	35.26	20.60
	BOD (mg/L)	1	26	7.39	5.25
	TSS (mg/L)	0.5	48	9.28	8.47
	TP (mg/L)	0.3	70	5.70	9.80
	TN (mg/L)	3	140	28.49	23.79
	NH_4-N (mg/L)	0.1	101	14.10	19.78
	NO_3-N (mg/L)	0.2	60	9.08	12.41

3.2.4 Microbiological analysis

Conventional microscopy

Activated-sludge wet mounts and smears were prepared for examination of filamentous bacteria. To determine morphological characteristics of the various filaments, wet mounts were inspected under bright-field and phase contrast (1000 X magnification microscopy). Smears were stained according to Gram and Neisser staining techniques and examined under oil immersion and bright-field (1000 X magnification). The sulphide oxidation test (S test) was applied to determine the presence/absence *in situ* of intracellular sulphur deposits to confirm the identifications of *Thiothrix* spp. and Type 021N. The microscopic observations of filamentous bacteria and their morphologic features were performed using an optical microscope CX41 (Olympus, Japan). Morphological identification of filamentous bacteria was done by comparing the observed filament with the various characteristics outlined by referenced manuals (Eikelboom 2000; Jenkins *et al.* 2004).

Fluorescence in situ hybridization

FISH was performed according to the standard protocol of Nielsen *et al.* (2009) to confirm the identification of some filamentous bacteria. The fixed samples were dehydrated in 50, 80, and 96 % (*v/v*) ethanol. After 2 - 3 h of hybridization at 46 °C, the samples were washed in a buffer and incubated for 10 - 15 min at 48 °C. Lysozyme and Achromopeptidase solutions were used to permeabilize Gram positive cells at 37 °C for 10 - 20 min. The probes were used to confirm identifications or to clarify certain morphotypes (Table 3.2), and the hybridization conditions applied (different formamide concentrations) were those detailed in their original publications (Table 3.2 – column: Source). The probes were fluorescently tagged with ALEXA488, Fluorescein, or CY3 fluorochromes. NONEUB338 probes labelled with ALEXA488, Fluorescein, and CY3 were used to confirm if non-specific binding occurred or if the fluorochrome adsorbed to the biomass. 4,6-diamidino-2-phenylindole (DAPI) staining was applied to visualize the morphology and distribution of filamentous bacteria within the biomass. The microscopic observations of filamentous bacteria were performed using an epifluorescence microscope BX51 (Olympus, Japan).

For some of the morphotypes probes are not available (e.g., Type 0961 and Type 0411) or clearly validated (e.g., Type 0803 and Type 0914). In what *Thiothrix* identification is concerned, two categories were considered. During FISH, some of the filaments previously identified as *Thiothrix* showed a negative response to the specific probe for *Thiothrix* spp. (G123T probe). The positive filaments were then termed "*Thiothrix* spp.". The other filaments had particular characteristics similar to the previously described *Thiothrix* spp. but showed a negative response to the S test and a positive response to the *Chloroflexi* group probe: they were referred as "*Thiothrix* - similar morphotype" in this study.

Table 3.2: FISH probes used in the present study

Probe	Morphotype	Sequence	Source
EUB338 I *	All Bacteria	GCTGCCTCCCGTAGGAGT	(Amann *et al.* 1990)
EUB338 II *	*Planctomycetales*	GCAGCCACCCGTAGGTGT	(Daims *et al.* 1999)
EUB338 III *	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	(Daims *et al.* 1999)
CHL1851	Type 1851 filamentous bacteria	AATTCCACAACCTCTCCA	(Beer *et al.* 2002)
G123T+G123T-C	*Thiothrix* spp.	CCTTCCGATCTCTATGCA+CCTTCCGATCTCTACGCA	(Kanagawa *et al.* 2000)
SNA	*S. natans*	CATCCCCCTCTACCGTAC	(Wagner *et al.* 1994)
HHY	*H. hydrossis*	GCCTACCTCAACCTGATT	(Wagner *et al.* 1994)
MPAmix (MPA60+MP223 +MPA645)	*M. parvicella*	GGATGGCCGCGTTCGACTGCCGCGAGACCCTCCTAGCCGGACTCTAGTCAGAGC	(Erhart *et al.* 1997)
Myc657	Nocardioform (GALO/PTLO)	AGTCTCCCCTGYAGTA	(Davenport *et al.* 2000)
NONEUB	Control probe complementary to EUB338	ACTCCTACGGGAGGCAGC	(Wallner *et al.* 1993)
CFXmix (GNBSB941 +CFX1223)	Phylum *Chloroflexi*	AAACCACACGCTCCGCT+CCATTGTAGCGTGTGTGTMG	(Björnsson *et al.* 2002); (Gich *et al.* 2001)
CF223+CFX197-C +CFX223	Type 0092 filamentous bacteria	TCCCGGAGCGCCTGAACT+TCCCGAAGCGCCTGAACT+GGTGCTGGCTCCTCCCAG	(Speirs *et al.* 2009)

* EUB338 I, II, and III used in equimolar amounts as EUBmix

3.2.5 Microbiological analysis quantification

Frequency is the number of times that each filamentous morphotype appears in the total set of samples. It was presented as a percentage of the occurrence in the 128 samples. Filamentous microorganisms were quantified according to a subjective scoring of filament abundance (0 = none, 1 = few, 2 = some, 3 = common, 4 = very common, 5 = abundant, and 6 = excessive) adapted from Jenkins *et al.* (2004). Abundance is weighed, corresponding to the sum of the products of the frequencies and the respective "scores" of abundances from 0 to 6. It was presented as a percentage of each weighed abundance in the sum of the weighed abundances of all morphotypes. The dominance corresponds to the number of times that a morphotype appears in "scores" of 5 and/or 6. It was also presented as a percentage.

3.2.6 Statistical analysis

In order to understand the relationships between the filamentous bacteria and the environmental parameters, correlations analyses were performed. These correlations were made using the most frequent morphotypes and only 78 samples were selected, since the other had missing data on environmental parameters (data not supplied by the WWTP).

Statistical data analysis, comparisons and graphing were performed in Microsoft Excel (USA), GraphPad Prism software 5.0 version (USA) and STATISTICA software 7.0 version (USA). Data statistical analysis was performed using STATISTICA. The non-normally distributed data were analysed using the non-parametric test Kendall rank correlation coefficient. Factor analysis (factor extraction method: principal component analysis (PCA), transformation method: Varimax) were also analysed using Kendall rank correlation coefficient. Statistically significant differences were determined for *p*-values ≤ 0.05(*), 0.01(**), and 0.001(***).

Fourteen physical-chemical and operational parameters of effluent quality were selected for data analysis: BOD_5, COD, TSS, pH, TN, NH_4–N, NO_3–N, and TP from the effluent and SRT, SVI, F/M, DO, MLSS, and pH from the aeration tank. Correlation coefficients were determined to examine potential relationships between the filamentous bacteria and quality of effluent. The Kendall rank correlation coefficient was used after experimental data were logarithmically transformed according to: $\boldsymbol{x = \ln(x + 1)}$.

3.3 Results and discussion

3.3.1 Microbiological analysis quantification

The frequency, abundance, and dominance of the filamentous bacteria in activated-sludge of the 16 WWTP during the sampling period are showed in Figure 3.1, Figure 3.2 and Table 3.3, respectively. 22 filamentous morphotypes were identified. Only the filamentous bacteria appearing in at least 50 % of the samples in terms of frequency were discussed. The most frequent filamentous morphotypes appearing in at least 50 % of the samples were, by this order, Type 0041/0675, Type 0092, *M. parvicella*, Type 1851, Nocardioforms, and *H. hydrossis*. The most frequent filamentous bacteria were also the most abundant, being the tendencies of both variables very similar, the only difference being the change in rank of *M. parvicella* and Type 1851. On the other hand, the most frequent and abundant were not the most dominant in the studied WWTP (Table 3.3). Type 1851 was clearly the most dominant (appearing with abundance "scores" of 5 and 6 in 13 % of the samples), followed by *M. parvicella* (10 %) and Types 0041/0675 and 0092 (9 %). These were also the most dominant filamentous bacteria in WWTP suffering bulking (SVI ≥ 150 mL/g) as shown in Table 3.4.

High frequencies of *M. parvicella* and Type 0041/0675 were observed by Blackbeard *et al.* (1986), Bux & Kasan (1994) and Lacko *et al.* (1999) that accredited success of these organisms to their ability to survive and to adapt to wide parameter ranges usual in activated-sludge systems. Eikelboom *et al.* (1998) stated that *M. parvicella* was clearly the most important filamentous species in nutrient removal plants, and Madoni *et al.* (2000) also observed that this species was the most frequent in foaming and bulking situations. In the present case, however, that was not observed: *M. parvicella* was the second in dominance (Table 3.3) but only the third in frequency and the fourth in abundance (Figure 3.1 and Figure 3.2, respectively). The importance of the Type 0092 was not surprising as this is a frequent morphotype in nutrient removal plants.

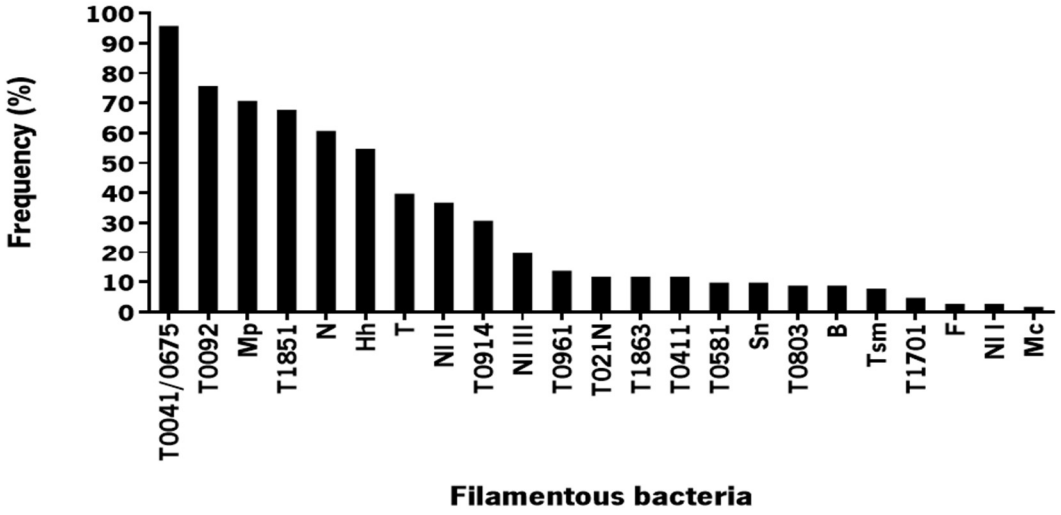

Figure 3.1: Frequency (% of occurence) of filamentous bacteria in the studied WWTP. **(T0041/0675)** Type 0041/0675; **(T0092)** Type 0092; **(Mp)** *M. parvicella*; **(T1851)** Type 1851; **(N)** Nocardioforms; **(Hh)** *H. hydrossis*; **(T)** *Thiothrix* spp.; **(NI II)** *N. limicola* II; **(T0914)** Type 0914; **(NI III)** *N. limicola* III; **(T0961)** Type 0961; **(T021N)** Type 021N; **(T1863)** Type 1863; **(T0411)** Type 0411; **(T0581)** Type 0581; **(Sn)** *S. natans*; **(T0803)** Type 0803; **(B)** *Beggiatoa* spp.; **(Tsm)** *Thiothrix* - similar morphology; **(T1701)** Type 1701; **(F)** *Flexibacter*; **(NI I)** *N. limicola* I and **(Mc)** *Microthrix calida*.

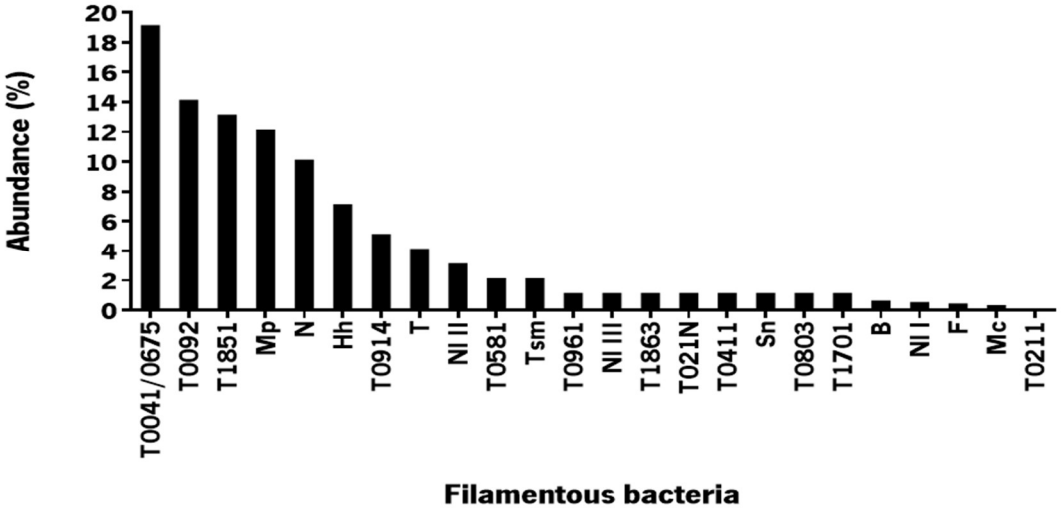

Figure 3.2: Weighed abundance (%) of filamentous bacteria in the studied WWTP. **(T0041/0675)** Type 0041/0675; **(T0092)** Type 0092; **(T1851)** Type 1851; **(Mp)** *M. parvicella*; **(N)** Nocardioforms; **(Hh)** *H. hydrossis*; **(T)** *Thiothrix* spp.; **(NI II)** *N. limicola* II; **(T0581)** Type 0581; **(Tsm)** *Thiothrix* **-** similar morphology; **(T0961)** Type 0961; **(NI III)** *N. limicola* III; **(T1863)** Type 1863; **(T021N)** Type 021N; **(T0411)** Type 0411; **(Sn)** *S. natans*; **(T0803)** Type 0803; **(T1701)** Type 1701; **(B)** *Beggiatoa* spp.; **(NI I)** *N. limicola* I; **(F)** *Flexibacter*; **(Mc)** *M. calida* and **(T0211)** Type 0211.

Table 3.3: Dominance of filamentous bacteria in the studied municipal WWTP

Dominant filamentous bacteria morphotype	Samples (%)
Type 1851	13
M. parvicella	10
Type 0041/0675	9
Type 0092	9
Thriothrix - similar morphology	4
Type 0581	3
Type 0914	2
Nocardioforms	2
Type 021N	1
S. natans	1

In the set of morphotypes with frequencies higher than 50 %, two were not among the most dominants (Table 3.3): *H. hydrossis* and Nocardioforms. *H. hydrossis* was a frequent and abundant filament (Figure 3.1 and Figure 3.2, respectively) that did not reach dominant positions in activated-sludge plants studied. It is to remember, however, that confirmations only took in consideration those filaments that hybridized with the probe HHY and other *H. hydrossis*-like filaments hybridize with other probes (Kragelund *et al.* 2008). Nocardioforms, on the other hand, were the fifth in the rank of the most frequent and abundant (Figure 3.1 and Figure 3.2, respectively) but were dominant in only 2 % of the WWTP samples (Table 3.3) and in 2 % of the bulking systems (Table 3.4). The relatively low importance of Nocardioforms was not expected (Eikelboom 1975; Jenkins *et al.* 2004; Rampersad 2002) but the fact that the samples of the present study were all coming from nutrient removal plants can account for the fact. Seviour & Nielsen (2010) reported that the most abundant filaments in nutrient removal WWTP treating mainly municipal wastewater were *M. parvicella*; followed by Types 0041/0675, 1851, and 1701; Type 0041 in TM7 division; *H. hydrossis*; Nocardioforms; Type 0092; and Type 0803. In what abundance concerns, the present results do not confirm these findings.

The fact that 97 out of the 128 samples correspond to bulking situations can account for the coincidence of dominance in the total of samples and in bulking samples. In these 97 samples, Type 1851 was the main responsible for the bulking (16 %), closely followed by *M. parvicella* (13 %), Type 0092 (11 %), and Type 0041/0675 (8 %) (Table 3.4).

Table 3.4: Dominant filamentous bacteria in WWTP samples with SVI ≥ 150 mL/g

Filamentous bacteria	Samples (%)
Type 1851	16
M. parvicella	13
Type 0092	11
Type 0041/0675	8
Thiothrix - similar morphology	5
Type 0581	4
Type 0914	2
Nocardioforms	2
Type 021N	1
S. natans	1

3.3.2 Statistical analysis

Results of the correlation analysis between the filamentous bacteria and the environmental parameters are presented in Table 3.5. Significant correlations between most of the filamentous morphotypes and several environmental parameters were obtained, with the exception of Type 0041/0675. These results corroborate Blackbeard *et al.* (1986), Bux & Kasan (1994) and Lacko *et al.* (1999) that stated its ability to survive and adapt to wide parameter ranges usual in activated-sludge systems.

Type 0092 (Table 3.5) showed significant correlations with four parameters. Since Type 0092 showed a positive correlation with pH (in both affluent and final effluent) and SVI and a negative correlation with organic load, this filamentous bacteria can be a useful indicator of poor sedimentation, low organic load, and relatively high pH in the plant. Madoni *et al.* (2000) also reported that Type 0092 was dominant only in plants with low F/M load. Eikelboom (2000) stated that this morphotype is largely present inside the flocs with limited effect on the SVI, but Blackbeard *et al.* (1986) reported these filamentous bacteria as dominant in bulking and foaming activated-sludge plants. The present results corroborate the latter. Many authors observed that Type 0092 appears in many filament bacteria surveys carried out on plants around the world (Martins *et al.* 2004; Tandoi *et al.* 2017), and it has been associated with long sludge age (SRT). This association was not observed in this study. This filament morphotype was classified as an "all-zone" grower by Wanner & Grau (1989), able to grow under aerobic, anoxic, and anaerobic conditions, and this can be the reason for the absence of correlation with DO.

Table 3.5: Correlations between the filamentous bacteria and the environmental parameters (Kendall correlations) in the 16 WWTP.

		Type 0041/0675	Type 0092	Type 1851	Nocardioforms	*M. parvicella*	*H. hydrossis*
Affluent	pH	-0.019	**0.165***	-0.040	**-0.190***	0.074	-0.055
	COD (mg/L)	-0.040	-0.090	-0.010	0.067	-0.112	-0.071
	BOD (mg/L)	-0.063	-0.042	-0.004	0.104	-0.122	-0.057
	TSS (mg/L)	-0.112	-0.054	-0.109	0.003	0.032	-0.089
	TP (mg/L)	0.114	-0.138	0.060	0.055	-0.029	0.026
	TN (mg/L)	0.020	-0.037	**-0.155***	-0.015	-0.003	**-0.159***
	NH_4-N (mg/L)	-0.048	0.028	**-0.176***	-0.108	-0.001	-0.143
Aeration tank	pH	-0.011	0.131	-0.085	**-0.193***	**0.176***	0.078
	SVI (mL/g)	0.114	**0.245****	**0.178***	0.069	0.021	-0.078
	SRT (d)	-0.035	0.122	0.120	**-0.208****	-0.145	0.023
	F/M (kg BOD_5/kg VSS.d)	-0.021	**-0.232****	0.020	**0.288*****	0.014	0.035
	DO (mg/L)	0.030	0.006	-0.018	0.075	0.056	-0.033
	MLSS (mg/L)	-0.047	0.015	-0.013	0.049	0.056	**-0.176***
Effluent	pH	-0.045	**0.277*****	-0.121	**-0.176***	**0.176***	0.117
	COD (mg/L)	-0.029	-0.070	-0.085	-0.024	0.044	0.096
	BOD (mg/L)	-0.008	0.056	0.038	0.015	**0.215****	0.074
	TSS (mg/L)	0.026	-0.028	-0.091	-0.002	0.124	0.032
	TP (mg/L)	0.034	0.091	-0.105	-0.142	-0.033	0.091
	TN (mg/L)	0.119	0.107	-0.141	-0.120	**0.168***	-0.002
	NH_4-N (mg/L)	0.115	0.050	-0.111	-0.052	**0.321*****	0.062
	NO_3-N (mg/L)	-0.064	0.116	**-0.220****	**-0.158***	-0.107	-0.112

* *p*-values ≤ 0.05; ** *p*-values ≤ 0.01; *** *p*-values ≤ 0.001

Type 1851 (Table 3.5) showed significant correlations with four environmental parameters of the affluent, mixed-liquor and final effluent. Type 1851 was also positively correlated with SVI and, as Type 0092, can be a good indicator of bad sedimentation of the sludge. This filament was dominant in urban plants receiving domestic and mixed sewage, contradicting several authors that referred its dominance in industrial but not in domestic plants (Seviour & Blackall 1999; Eikelboom 2000), namely in the presence of simple sugars and soluble starches (Jenkins *et al.* 2004). The negative correlation with sewage TN and NH_4–N and effluent NO_3–N, suggests that Type 1851 is associated with N deficiencies, although these associations were never reported in previous studies.

Nocardioforms (Table 3.5) showed the highest number of correlations: with the affluent, mixed-liquor (aeration tank) and effluent pH, the SRT, the F/M (being this the only positive correlation with Nocardioforms), and the NO_3-N in the effluent. Nocardioforms were positively associated with F/M, supporting others works that found Nocardioforms in a wide range of broad organic loads (Madoni *et al.* 2000; Jenkins *et al.* 2004). Consequently, they were negatively correlated with sludge age (SRT). Also, Nocardioforms increased when the pH in the aeration tank decreased. Madoni *et al.* (2005) reported that the optimum pH for Nocardioforms is between 7 and 8, and their growth rate decreases and attains a minimum at pH 5, contradicting the results of the present study. In contrast, Spigoni *et al.* (1992) reported that *Nocardia* spp. is favoured by relatively low pH (6.5 - 7.1). As the studied plants are all nutrient-removal WWTP, the negative correlation with the final effluent nitrate level, also observed with the Type 1851, can mean that these two morphotypes are associated with good denitrifying conditions.

The presence of *M. parvicella* (Table 3.5) was positively correlated with the pH in the aeration tank and in the final effluent. Spigoni *et al.* (1992) stated that *M. parvicella* in pure culture grows at an optimum pH between 7 and 8, whereas in the activated-sludge is also found at lower pH, controverting the association found in this study. Positive correlations were also found between this filament and BOD_5, TN, and NH_4–N in the final effluent. This can simply mean that the abundance of *M. parvicella* may cause the washout of biomass, explaining the higher content of N and organic matter in the effluent.

H. hydrossis (Table 3.5) exhibited only two significant negative associations: with MLSS and TN in sewage (affluent). The negative correlation between *H. hydrossis* with N in the affluent was not expected, since these filamentous bacteria are associated with high concentration of N compounds (Eikelboom 2000; Madoni *et al.* 2000). The negative correlation with MLSS is not mentioned in other works. It was expectable that *H. hydrossis* would show a negative association with oxygen in aeration tank (Wanner &

Grau 1989; Seviour & Blackall 1999; Jenkins *et al.* 2004), but no associations were found in the present study.

Table 3.6 shows the associations obtained by PCA between the filamentous bacteria species and some of the environmental parameters from the activated-sludge in the 16 WWTP. Nine factors were extracted through factor analysis, explaining 73.636 % of the total variance. In order to make each factor effective, the percentile explained by variance should be more than 10 %. With this criterion, only three factors were effective (factor 1 to factor 3). Due to the low percentage of explained variables, the three factors defined account for 39.482 % of accumulated variances. The other six factors (factor 4 to factor 9) might be useful through relating with correlation coefficients present in Table 3.5. The factor loadings, also called component loadings in PCA, are the correlation coefficients between the variables (biological and environmental parameters) and factors. The significant values for these factor loadings are above 0.60 according to Hair *et al.* (1995).

Table 3.6: Factor analysis of the filamentous bacteria and the environmental parameters in the 16 WWTP

		Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9
Filamentous bacteria	Type 0041/0675	-0.042	0.112	-0.117	**0.772***	0.006	0.204	-0.033	0.023	0.061
	Type 0092	-0.101	-0.274	0.013	0.058	**-0.731***	0.182	0.262	-0.010	-0.112
	Type 1851	-0.057	0.081	-0.027	**0.614***	-0.316	-0.357	-0.129	0.053	-0.102
	Nocardioforms	0.171	0.159	-0.125	0.557	0.058	-0.191	-0.201	0.126	0.423
	M. parvicella	-0.156	0.010	0.249	0.047	-0.321	0.030	0.461	0.251	0.385
	H. hydrossis	-0.061	-0.180	0.238	**0.700***	0.073	-0.039	0.242	-0.348	-0.113
Affluent	pH	0.198	0.294	0.075	-0.204	-0.489	0.198	0.406	-0.047	-0.307
	COD (mg/L)	**0.912***	-0.010	0.161	0.019	-0.008	0.078	-0.064	0.018	0.025
	BOD_5 (mg/L)	**0.951***	-0.014	0.043	0.041	-0.007	-0.034	-0.065	0.022	0.022
	TSS (mg/L)	**0.872***	-0.073	0.136	-0.073	0.039	0.159	0.017	0.078	0.127
	TP (mg/L)	0.101	**0.749***	0.091	0.206	0.119	-0.079	-0.207	-0.086	-0.022
	TN (mg/L)	0.579	0.119	-0.291	-0.104	0.083	0.424	0.114	0.186	-0.125
	NH_4-N (mg/L)	0.368	-0.463	-0.162	-0.206	0.034	0.462	0.185	0.356	-0.143
Aeration tank	pH	-0.142	0.065	0.022	-0.072	0.054	-0.135	**0.824***	-0.173	-0.053
	SVI (mL/g)	0.001	0.201	-0.123	0.061	**-0.821***	-0.149	-0.190	-0.040	-0.022
	SRT (d)	-0.051	-0.034	-0.095	0.018	-0.100	0.018	0.080	0.015	**-0.831***
	F/M (kg BOD_5/kg VSS.d)	0.348	0.188	0.331	0.067	0.216	-0.214	0.026	-0.383	0.498
	DO (mg/L)	-0.135	**0.683***	0.136	-0.086	-0.185	0.132	0.197	-0.044	0.120
	MLSS (mg/L)	0.188	-0.182	0.178	-0.059	0.042	-0.134	-0.121	**0.765***	-0.016
Effluent	pH	0.106	-0.193	-0.042	-0.011	-0.086	0.168	**0.815***	-0.001	-0.113
	COD (mg/L)	0.225	0.148	**0.760***	-0.047	0.068	0.097	-0.017	0.036	-0.090
	BOD_5 (mg/L)	0.090	0.099	**0.786***	0.120	-0.043	0.024	0.099	0.169	0.116
	TSS (mg/L)	-0.055	-0.005	**0.815***	-0.139	0.045	0.086	0.025	-0.027	0.136
	TP (mg/L)	0.213	-0.088	0.155	0.092	0.057	**0.739***	0.006	-0.056	-0.174
	TN (mg/L)	-0.006	0.166	0.216	0.045	-0.036	**0.719***	0.332	0.203	0.011
	NH_4-N (mg/L)	0.022	0.169	0.273	0.106	0.117	0.392	0.517	0.424	0.099
	NO_3-N (mg/L)	0.065	-0.026	-0.063	-0.214	-0.177	**0.773***	-0.210	-0.341	0.075
Variance explained (%)		16.017	12.446	11.018	7.504	6.619	5.993	5.274	4.598	4.166
Accumulated variance (%)		16.017	28.464	39.482	46.985	53.605	59.598	64.872	69.470	73.636

* statistically significant

Figure 3.3 shows the scatter diagrams of the first three and the fifth factors. The first principal component (factor 1) explains 16.017 % of the total variance. This factor was related with organic matter variables (COD, BOD_5, and TSS) in plant affluent, and they were correlated positively with each other, which is expectable.

The second factor, accounting for 12.446 % of the total variance, was related with TP in sewage and DO in the aeration tank.

The third factor, explaining 11.018 % of the total variance, was also positively correlated with organic matter variables (COD, BOD_5, and TSS) but in this case, in the final effluent.

The biological data, such as Type 0041/0675, Type 1851, and *H. hydrossis* were positively associated with each other, meaning that they were expected to appear together, constituting the fourth factor (7.504 % of the total variance). Eikelboom (2000) described that *H. hydrossis* and Type 0041/0675 populations are always small in domestic WWTP with nutrient removal, so they can appear together. The presence of both *H. hydrossis* and Type 1851 was also detected in an influent from agro-industry (Eikelboom 2000). Although, *H. hydrossis* are present in high F/M and Types 0041/0675 and 1851 are present in low F/M, so there is not a condition that all have in common, which contradicts the results of PCA.

The other factors were more related with environmental parameters, except for the fifth factor, accounting with 6.619 % of the total variance, showing that Type 0092 had a relation with SVI - the only significant PCA association between biologic and environmental parameters - corroborating the correlation found in Kendall correlation analysis (Table 3.5).

PCA can be a useful method for reducing the number of variables to be taken in account in a data set by finding linear combinations of those variables that explain most of the variability. In the present case, PCA was of limited value but presented some expected associations among some environmental parameters (COD, BOD_5, and TSS in entrance and exit) and among some of the filamentous morphotypes. It also reaffirmed the association between Type 0092 and SVI.

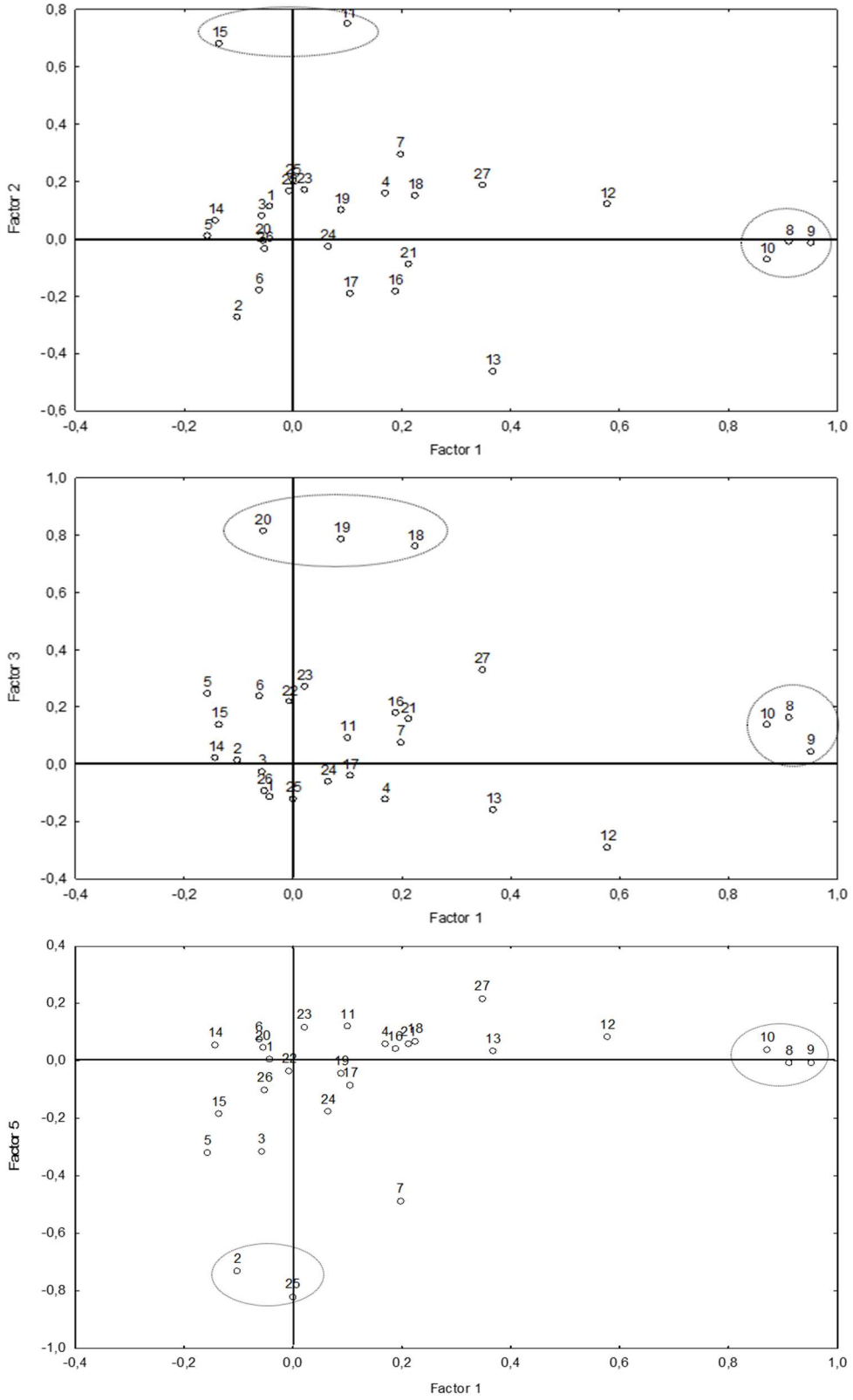

Figure 3.3: Distribution of the filamentous bacteria and environmental parameters as defined by the first three factors of the principal component analysis (PCA). **(1)**Type 0041/0675; **(2)** Type 0092; **(3)** Type 1851; **(4)** Nocardioforms; **(5)** *M. parvicella*; **(6)** *H. hydrossis*; **(7)** pH affluent; **(8)** COD affluent; **(9)** BOD affluent; **(10)** TSS affluent; **(11)** TP affluent; **(12)** TN affluent; **(13)** NH_4-N affluent; **(14)** pH aeration tank; **(15)** DO aeration tank; **(16)** MLSS; **(17)** pH effluent; **(18)** COD effluent; **(19)** BOD effluent; **(20)** TSS effluent; **(21)** TP effluent; **(22)** TN effluent; **(23)** NH_4-N effluent; **(24)** NO_3-N effluent; **(25)** SVI; **(26)** SRT and **(27)** F/M.

3.4 Conclusions

A diverse community of filamentous bacteria inhabits the activated-sludge process. Type 0041/0675, Type 0092, *M. parvicella*, Type 1851, Nocardioforms and *H. hydrossis* were more frequently observed and were more abundant than any other filamentous bacteria in the 16 studied WWTP.

Differing from frequently and abundant results, substantial differences were found in the dominance: Type 1851 and *M. parvicella* were the most dominant filamentous bacteria in all studied WWTP, followed by Types 0041/0675 and 0092. These morphotypes were exactly those that revealed to be problematic: they were the dominant morphotypes during the bulking occurrences in WWTP. These results reinforce the need to clarify the meanings of frequency, abundance and dominance when discussing the causes of bulking in WWTP.

The Kendall correlation analysis showed that the prevalence and abundance of filamentous bacteria can be influenced both by wastewater composition, such as pH, TN and NH_4-N, and by process design/operational parameters, such as SVI, SRT and F/M.

In this study, PCA with correlation coefficients did not help to reaffirm the relationship between filamentous bacteria and most of operational parameters, apart from the positive correlation between Type 0092 and SVI, but confirmed correlations between environmental parameters and between filamentous bacteria.

Overall, the results of the present study, including the different ranges of the various morphotypes when considering frequency, abundance, and dominance, and the contradictions among results published by other authors, emphasize the importance of distinguishing between frequency, abundance, and dominance of filamentous morphotypes in domestic and industrial WWTP, including the correlations with environmental parameters.

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CHAPTER 4

In-vitro assessment of biocides effect on specific filamentous bacterial populations

In this chapter, the *in-vitro* effect of biocides on two filamentous bacteria strains was assessed. The growth of two filamentous bacteria in pure cultures, *Nocardia amarae* and *Sphaerotilus natans*, was assessed with three biocides: triclosan, CTAB and glutaraldehyde. The XTT reduction assay results showed a dose-dependent effect on bacterial viability for all the three compounds. Differences in the susceptibility to the exposure to each biocide suggested strain-dependent effects. CTAB was the most toxic compound among the three used to *N. amarae* and the intermediate toxic when considering the assays with *S. natans*. Regarding the cytometry assay, 48 h after biocide removal, the results showed that both strains had not recovered from it in any of the three cases, being glutaraldehyde the biocide with most prolonged toxicity in the case of *N amarae*. However, CTAB showed the more pronounced effects in both strains. Both strains fragmented their filaments to single cells and the ability to produce flocs was lost, at least until 48 h after the biocide removal, in the three cases. The differences observed in the toxicity effects corroborate the existence of different mechanisms of action of the three biocides and, on the other hand, must be taken in consideration when the overgrowth of a particular strain into be controlled.

This chapter is based on the following submitted publication: Ferreira, V., Dias, N., Mota, M., Pereira, M.O., Nicolau, A., 2022 *In-vitro* assessment of biocides effect on specific filamentous bacterial populations. (Submitted to *International Journal of Environmental Research and Public Health*).

4.1 Introduction

Activated-sludge process is the most widely used technique for wastewater treatment. The bacterial populations of activated-sludge are characterized by floc-forming and non-floc-forming organisms, being the latter mostly filamentous bacteria. In WWTP, the sedimentation and thickening processes usually fail due to the overgrowth of filamentous species in a phenomenon known as filamentous bulking (Nittami *et al.* 2019). Filamentous bacteria can also be the source of another WWTP malfunction characterized by the formation of foams. Among filamentous bacteria, *S. natans* is one of the causes of bulking whereas *N. amarae* is frequently associated to the foaming phenomenon but also to bulking episodes in activated-sludge systems (Seviour & Nielsen 2010; Bafghi & Yousefi 2016). *S. natans* is a Gram negative bacteria associated with low nutrient systems and/or with low oxygen content. The most effective corrective measure to control the growth of this species seems to be the increase of aeration (Seder-Colomina *et al.* 2015). *N. amarae* is a Gram positive bacteria, associated with high temperatures and influents rich in fats and oils (Pal *et al.* 2014; Tandoi *et al.* 2017). The main strategy for solving the excessive growth of this species is reducing the residence time in the aeration tank.

Filamentous bulking can be controlled by specific and/or non-specific methods. Specific methods aiming at recognizing and resolving the major cause of the filamentous bacterial proliferation episode and are preferred, because they are selective for the target microorganism and do not damage the remaining biomass (Henriet *et al.* 2017). In what concerns non-specific methods, selective toxicants are used, although these substances are only temporary solutions and tend to damage floc-forming bacteria, leading to process breakdown in the long run (Caravelli *et al.* 2007). Chlorination was one of the first methods used to control filamentous bulking and it is still used (Tandoi *et al.* 2017), even though alternatives are nowadays available. The addition of biocides is one of the most commonly used methods under critical conditions to improve the sedimentation process. Biocides have a diversity action mechanism that can be applied through attack of the cell wall (cell viability), the cytoplasmic or cellular membrane (Denyer 1995) and its susceptibility varies between Gram positive and negative bacteria, being Gram negative bacteria generally reported to be less susceptible to biocides (Wales & Davies 2015). Nevertheless, Caravelli *et al.* (2007) reported that the surfactant Triton X-100 inhibited *S. natans* (Gram negative) growth inducing its cell lysis and Guo *et al.* (2012) described the effect of CTAB on chlorine-resistant Type 021N (Gram negative), due to its stronger penetration capacity through the cell wall.

In this study, biocides from three different chemical groups were chosen as effective biocidal agents against bacteria. Triclosan, as an oxidizing biocide belonging to the phenolic compound group, (Figure

4.1 (a)) is a widely used broad-spectrum bactericide, due to its effectiveness and thermal stability; it is found in a wide range of consumer products, namely toothpaste, antibacterial soaps, dishwashing liquids, cosmetic and antiseptic products (Carey & McNamara 2015). Triclosan is only sparingly soluble in water (10 mg/L) but it is soluble in solutions of dilute alkalis and organic solvents (Fraise *et al.* 2013). At low concentrations, (0.01 µg/mL to 0.1 µg/mL in water) it inhibits bacterial lipid biosynthesis by blocking the enoyl-acyl carrier protein reductase (ENR), the target sites for its action in some microorganisms (Dhillon *et al.* 2015). CTAB is a cationic surfactant (non-oxidizing biocide belonging to QACs) (Figure 4.1(b)) and one of the components of the typical antiseptic cetrimide (Guo *et al.* 2012). CTAB is an effective biocidal agent against bacteria and its action was previously reported against filamentous bacteria (Bratby 2016; Seka *et al.* 2001), improving the settling of activated- sludge systems. The mode of action is attributed to the positive charge, which forms an electrostatic bond with negatively charged sites on microbial cell walls (Simões *et al.* 2006). Glutaraldehyde (an aldehyde-based biocide) is also a non-oxidizing antimicrobial agent (Figure 4.1 (c)) and is widely used in a variety of applications, against bacteria and their spores (but not only) (Yoo 2018). Among other aldehydes, glutaraldehyde has been the most important antimicrobial agent (Russell 2002). Schuppler *et al.* (1998) reported a fixation protocol where glutaraldehyde promotes cell wall permeability, for penetration by the FISH probes in Nocardioform actinomycetes. Glutaraldehyde biocidal activity involves the cross-linking of the outer proteinaceous layers of the cell in such a way that cellular permeability is altered (Laopaiboon *et al.* 2006; Yoo 2018).

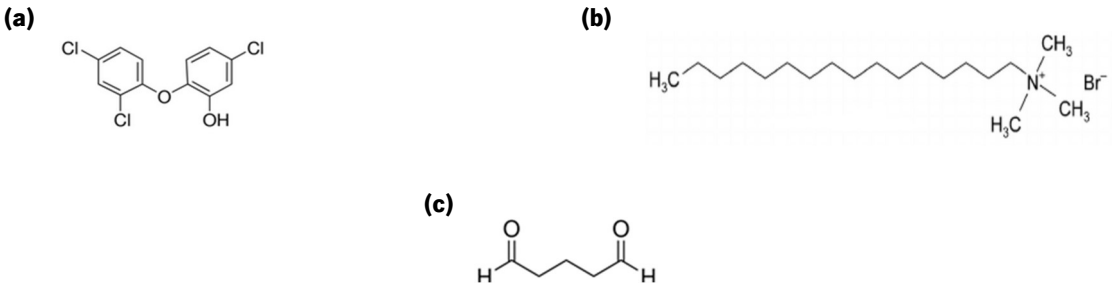

Figure 4.1: Biocides structure: **(a)** Triclosan (adapted from Fraise *et al.*, 2013); **(b)** CTAB (adapted from Avramova *et al.*, 2007) and **(c)** Glutaraldehyde (adapted from Jobish *et al.*, 2012).

Bacterial viability is the survival and reproductive capacity, typically assessed by plate counting (Auty *et al.* 2001). The plate count method is a traditional method used to assess the viable microorganism growth (quantitative survival). The total number of cells is estimated by counting colonies, where theoretically each colony develops from a single cell (Ou *et al.* 2017). It is considered the only direct proof of culturability; however, this method has inherent drawback, when used as an indicator of viability. Besides

being labour intensive, cell clumping and long incubation times (days to few weeks) are referred as some of the disadvantages (Bunthof *et al.* 2001). These drawbacks greatly limit the routine application of plate counts for determining rates of microbial inactivation, rapid screening of biocide efficacy, and rapid screening of resistant microorganisms. In consequence, estimation of viability based on the capacity of organisms to divide and form colonies have been questioned (Roslev & King 1993).

One commonly used standard method for assessing the culture status is the measurement of the optical density (OD) of growing cultures. OD continuous measurement is the most basic and powerful tool to provide optimal yields (total microbial growth) and controlling reproducibility (Coburn 1979). Nevertheless, this method does not differentiate between live and dead bacteria, and, therefore, has low sensitivity. Furthermore it is limited to concentrations between 10^8 and 10^{10} bacteria/mL (Hazan *et al.* 2012).

A variety of cell-based assays has been used to estimate the number of viable eukaryotic cells. Some of the major methods use multi-well formats where data are recorded using a plate reader, are often used for screening collections of compounds to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death (Riss *et al.* 2004). Tetrazolium salts have become some of the most widely used tools for measuring the metabolic activity of cells ranging from mammalian to microbial origin (Berridge *et al.* 2005; Rai *et al.* 2018). It requires incubation of a reagent with a population of cells or microorganisms. The viable population converts a substrate to a coloured product that can be easily quantified by colour change with a plate reader. Under most standard culture conditions, it will generate a signal that is proportional to the number of viable cells. Non-viable (or dead) cells rapidly lose the ability to convert the substrate to a formazan product. That difference provides the basis for many of the commonly used cell viability assays. Tetrazolium salts have previously been used to determine the metabolic rates of higher eukaryotic cells and to assess the effects of cytotoxic agents, to provide a non-destructive and continuous spectrophotometric measurement of cell respiration (Mosmann 1983; Stowe *et al.* 1995). The tetrazolium salt can be quantitatively reduced by dehydrogenase to a highly-coloured formazan crystals (colorimetric indicator of viability). Formazan crystals can be either observed microscopically in the cell cytoplasm or extracted and dissolved with organic solvents, such as dimethylsulfoxide (DMSO), enabling spectrophotometric quantification (Hatzinger *et al.* 2003). The tetrazolium salt 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) is the compound commonly used to measure dehydrogenase activity in *in-vitro* evaluation of cellular proliferation and cytotoxicity (Dias *et al.* 1999; Scholes *et al.* 2016). The reduced product of

MTT is not soluble in culture medium and therefore 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) become a better option for a quick assay (Rampersad 2012). The XTT assay is also a semi-quantitative assay based on the ability of mitochondrial dehydrogenases of viable cells to convert the tetrazolium salt into a reduced formazan-colored product. To give satisfactory colour accumulation the addition of an electrocoupling agent is required, such as phenazine methosulphate or menadione (Roslev & King 1993). Unlike MTT, the reaction with XTT results in a soluble formazan dye. The formazan-coloured product can be easily quantified without performing additional steps such as centrifugation, addition of lysis buffer, solubilisation, removal of medium, and sonication (Ramage *et al.* 2001). Thus, this eliminates a final solubilisation step, which means less manipulation and consequently, a reduced risk of error. Correlation between cell number and the amount of formazan produced allows cell respiration to be assessed quickly (Stowe *et al.* 1995; Pérez *et al.* 2010). In this study, it was hypothesised that metabolically active (viable cells) would produce a colour change, whereas non-metabolically active or non-viable cells, due to biocide treatment, would not. A more direct approach to estimate cell viability may be the use of fluorescence-based techniques, such as, epifluorescence microscopy, and more recently, flow cytometry (Keer & Birch 2003). Fluorescence microscopy has the advantage of allowing a rapid and direct assessment of cell viability despite individual strains being impossible to identify. In the review of Kepner & Pratt (1994) direct counting by epifluorescence was described as a suitable method for enumeration of total bacteria in environmental samples.

Flow cytometry can rapidly provide information about proliferation, thus allowing the assessment of population heterogeneity because it measures numbers, response to fluorescence probes or dyes and geometries, that is, rapid *in situ*, analysis of individual microorganisms (Veal *et al.* 2000; Patakova *et al.* 2014). Flow cytometry is an appealing technique for fast viability assessment, that can be assessed at the single cell level without culturing cells (Berney *et al.* 2007; Bunthof *et al.* 2001). On other words, flow cytometry permits simultaneous measurement of multiple cellular parameters, both structural and functional (O'Donnell *et al.* 2013), which are usually based on light scattering and fluorescence. However, it requires differentiation between live and dead bacteria. A membrane impermeant fluorescent probe that can passively diffuse through the cell wall can act as an indicator of non-viability, representing a loss in membrane integrity (Lebaron *et al.* 1998). On the other hand, membrane integrity analysis can be based on cells ability to exclude compounds such as fluorescent intercalating dyes, like propidium iodide (PI) (Kainz *et al.* 2017). The enumeration of dead (non-viable and membrane permeable) cells is not enough to distinguish from the effect of biocides on live (viable and membrane impermeable) and injured (viable but membrane permeable) microorganisms. A combination of fluorescent dyes must be used for

this purpose. The SYTO family dyes (Molecular Probes) penetrates viable, injured and non-viable bacteria and stains green, while PI penetrates bacteria with damaged cell membranes only, quenching the green SYTO fluorescence, thus staining dead bacteria red (Netuschil *et al.* 2014). Bacterial cells with compromised membranes (injured) emit red and green fluorescence or the combination of the two colours, as orange (Falcioni *et al.* 2008).

In this study, the biocidal effect of different concentrations of biocides, namely triclosan, CTAB, and glutaraldehyde, against specific filamentous, *N. amarae* and *S. natans*, was evaluated using different methods to estimate viable and non-viable bacteria in pure cultures and also the filamentation ability.

When assessing the biological consequences of xenobiotics in *in-vitro* experiments, dose is a central parameter (Doskey *et al.* 2015). Various dose-metrics are usually used including nominal concentration, total concentration, freely available concentration, but usually in terms of mass of the compound *versus (vs)* the volume of the culture or the number of cells. Traditional metrics are mg/L, mg/mL, and so on, but recent tendency is the use of moles of the assessed compound *vs* the volume of the culture or the number of cells. In fact, each site in the cell will be affected by a molecule and not by a microgram (or other mass unit) of the substance. In the present case, the study was initially conceived with µg/mL metrics but conclusions are withdrawn based also on µM.

4.2 Experimental methods and procedures

4.2.1 Filamentous bacteria strains

Pure cultures of filamentous bacteria, *Gordonia amarae* (*Nocardia amarae*) (DSMZ 43392) and *Sphaerotilus natans* (DSMZ 6575), were used. These bacteria were selected among a few activated sludge-inhabiting available strains, taking in account that the source should be activated-sludge systems and of different GRAM responses. Bacteria were cultured as described in the catalogue of strains from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *N. amarae* was grown in GYM Streptomyces medium 65 (containing, per liter, 4 g of Glucose, 4 g of Yeast extract, 10 g of Malt extract, 2 g of calcium carbonate ($CaCO_3$) and 12 g of Agar ($CaCO_3$ and Agar are used only in the case of solid growth medium) [pH 7.2]) and *S. natans* was grown in *Sphaerotilus* medium 51 (containing, per liter, 5 g of beef extract and 15 g of Agar (to prepare the solid growth medium) [pH 7.0]). The culture media were autoclaved at 120 °C for 20 min. The *N. amarae* and *S. natans* cultures

were grown in 100 mL Erlenmeyer flasks, incubated in an orbital shaker at 120 rotation per minute (rpm) at 25 °C and 28 °C, respectively. Aseptic techniques were used for all subsequent procedures.

Biomass concentration was determined by absorbance (λ) measurements at 600 nm and a growth curve was previously determined. OD measurements were carried out with Genesys 20 Visible Spectrophotometer (Thermo Scientific, USA). The growth curve was obtained and the nonlinear equation was fitted with the Boltzmann sigmoidal model (Prism software 5.0 version, USA). The following equation (Eq. 4.1) describes the bacterial growth considering a constant doubling time at the exponential phase:

$$\boldsymbol{y = y_0 e^{kx}} \qquad \text{Eq. 4.1}$$

where y_0 is the y value at time 0; k is the growth rate constant, expressed as h^{-1}. The time required for doubling the bacterial population (doubling time) at the exponential phase is expressed as T_d:

$$\mathrm{T_d} = \frac{\ln 2}{\mathrm{k}}$$

4.2.1.1 Growth curves for the bacterial strains

A growth curve representing the optical density (OD) measured at 600 nm was plotted as a function of incubation time (h) at 25 °C and 28 °C for *N. amarae* (Figure 4.2 (a)) and *S. natans* (Figure 4.2 (b)), respectively. Three distinct phases were found, corresponding to the three-phase linear model described by Buchanan *et al.* (1997). During the lag phase, no net increase in cell number was observed, as bacterial cells need to adapt to the new environment. *N. amarae* showed a longer phase of adaptation than *S. natans*. At the exponential phase, cells were rapidly growing and dividing at maximal growth rate with a doubling time of 3.429 h and 6.385 h for *N. amarae* and *S. natans*, respectively. As bacterial cell number increased, the limitation of nutrients and the toxic waste product accumulation became more significant. After the complete consumption of nutrients, either the bacterial cells arrested dividing or the rate of division matched the rate of cell death, resulting in the stationary phase. *S. natans* attained the stationary phase at 25 h to 30 h, about 10 hours before *N. amarae* (40 h).

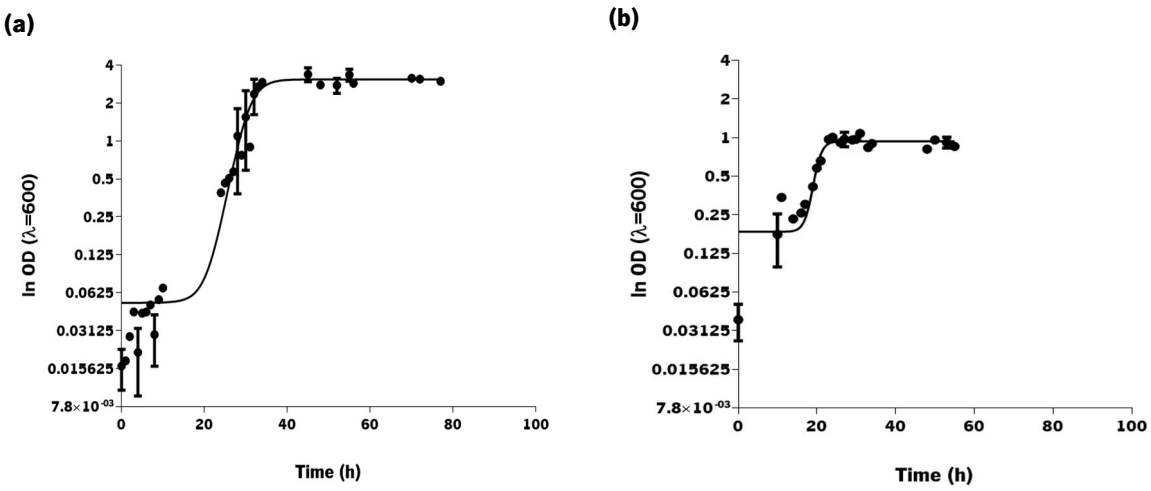

Figure 4.2: Natural logarithm of OD *vs* time (h) representing the growth curve of **(a)** *N. amarae and* **(b)** *S. natans.*

4.2.2 Biocides

Triclosan (97 % pure) was purchased from Aldrich Chemicals (USA). The triclosan stock solution was prepared in DMSO (8 mg/mL). CTAB was purchased from Acros Organics (USA) (99% pure) and the stock solution was prepared in distilled water (8 mg/mL of CTAB in distilled water). Glutaraldehyde solution 25 % in H_2O (density 1.062 g/mL) was purchased from Aldrich Chemicals – corresponding to the stock solution.

A preliminary XTT assay was performed as a screening test to select the more suitable range of concentrations of the biocide, in order to obtain a range of different effects. Initially, two times of sampling were chosen, 24 and 48 h, but as the cultures were already in the stationary phase in the second point, complicating the establishment of cause-effect relationships, it was decided to discard the second point in the definitive assays. So, one control (without biocide) and six different concentrations biocides (seven in the case of triclosan) were tested in a single end-point assay at 24 h (Table 4.1). Three independent assays were performed, and sampling was made in triplicate. The biocides were added at the beginning of the test (time = 0 h). In the Live/Dead viability assays, an extension to 72 h was carried on to assess the recovery of the biocidal effects.

Table 4.1: Single end-point assay concentrations (µg/mL)

Biocide	*N. amarae*							
Triclosan	0	10	20	40	60	80	120	200
CTAB	0	1	2	4	8	20	40	-
Glutaraldehyde	0	80	120	200	400	800	1000	-
Biocide	*S. natans*							
Triclosan	0	0.25	0.5	1	2	4	8	-
CTAB	0	1	2	4	8	20	30	-
Glutaraldehyde	0	40	50	75	80	120	200	-

4.2.3 *In-vitro* assessment methods

4.2.3.1 XTT reduction assay

The viability of bacteria was assessed by XTT assay, which was based on the experimental protocol described by Pettit *et al.* (2005). The protocol was adapted to filamentous bacteria strains and experimental conditions. The sodium salt of XTT (Sigma-aldrich, USA) was dissolved in PBS to 1 mg/mL. Menadione (Sigma-aldrich, USA) was dissolved in acetone to 1 mM. The XTT/Menadione reagent was prepared fresh prior to each assay and contained 12.5 parts XTT/1-part Menadione.

Biocides were added on each experimental well of a 96 well-microplate (except in culture and DMSO controls) to obtain the single end-point concentrations (Table 4.1). Fresh inoculum was obtained from a 24 h grown culture and OD at 600 nm was adjusted to 0.5 (cells exponential phase - Figure 4.2 (a) and (b)) for both strains and 100 µL was inoculated to the wells. After 24 h, 50 µL of XTT/Menadione was added to a final volume of 250 µL and the plates were incubated in the dark for 4 h at 28 °C, at 50 rpm. The absorbance at 490 nm was read on a microplate reader (Synergy HT, BioTek Instruments Inc., USA). Table 4.2 represents the controls used in each assay. The medium negative control was used to assess the colour interference of the biocide when dissolved into the culture medium. The culture negative control was used as a control for non-viable (dead) inoculum, which was heat treated at 120 °C for 15 minutes. Culture positive control was used to determine 100 % of viability. DMSO was used as a solvent for triclosan. As DMSO is known to affect cell viability in concentrations higher than 1.5 % (Chen & Thibeault 2013; Yuan *et al.* 2014), a DMSO control (DMSO < 2.5 %) was performed to assess the potential damaging effect of DMSO on the inoculum.

Table 4.2: Controls used in each assay

	Culture medium	Inoculum	Biocide	XTT/Menadione
Medium negative control	Yes	No	Yes	Yes
Culture negative control	Yes	Yes (heat treated)	No	Yes
Culture positive control	Yes	Yes	No	Yes
DMSO control	Yes + DMSO*	Yes	No	Yes

* Concentration of DMSO up to 2.5%

An increase in the number of live (respiring) bacteria correlated with an increase in overall activity of the dehydrogenases responsible for transforming the sodium salt of tetrazolium XTT into formazan, and viability was calculated using the following equation (Eq. 4.2):

$$\%\ \boldsymbol{viability} = \frac{(\boldsymbol{OD_{experimental\ well} - OD_{blank}}^{*})}{\boldsymbol{OD_{culture\ positive\ control}}} \times \mathbf{100} \qquad \text{Eq. 4.2}$$

$$^{*}\ OD_{blank} = (OD_{medium\ negative\ control}) - (OD_{culture\ negative\ control})$$

Lethal concentration 50 (LC_{50}) is a measure of toxicity of a substance found in the environment and is usually experimentally determined by exposing a tested population to a toxicant (Jeyaratnam *et al.* 2016; Khan *et al.* 2007). Bacterial mortality was expressed as LC_{50} which defines the biocide concentration at which 50 % of the bacterial population died after 24 h of exposure. Linear regression analysis was used to determine LC_{50} values for triclosan, CTAB and glutaraldehyde.

4.2.3.2 Live/dead viability assay (Cytometry and Epifluorescence microscopy)

Dyes and staining conditions

SYTO-bacterial count (SYTO-BC) and PI were purchased from Molecular Probes, Invitrogen, Eugene, OR, USA. SYTO-BC stock solution was prepared at 5 µM in pre-filtered (0.2 µm) sterilized water and PI was used at 1.0 mg/mL. Stock solutions were stored frozen at –20 °C and protected from light.

Bacterial preparation and staining conditions

A volume of 2 mL of an exponential phase the bacterial culture was concentrated by centrifugation at 10,000 × g for 1 minute. The supernatant was removed and the pellet was re-suspended in 2 mL of pre-

filtered 0.85 % sodium chloride (NaCl) buffer. The bacterial cells were pelleted again by centrifugation at 10,000 × g for 1 minute. The supernatant was removed and the pellet was re-suspended in 500 µL of pre-filtered 0.85% NaCl buffer. The samples of heat-treated bacterial cells were heated at 120 °C for 15 minutes.

For the live/dead staining viability assay, dyes were added to the bacterial suspension at a final concentration of 0.30 µM of SYTO-BC and 1.5 µM of PI.

Flow cytometry

Single-colour controls for live and dead cell bacterial suspensions were used to set up the flow cytometer EC800 Flow Cytometry Analyzer (Sony Biotechnology Inc., USA) equipped with a blue laser (488 nm). In addition, fluorescence signals were detected by two photomultiplier tubes with band-pass filters (BP) for green fluorescence (FL1) emission spectra (EM) BP525/50 and for red fluorescence (FL4) EM BP 665/30. Green fluorescence (SYTO-BC) was detected in channel FL1, and red fluorescence (PI) in channel FL4. A flow rate of 10 µL/min, with a sample volume of 150 µL and 20000 counts/sample were set as parameters for data acquisition. Every sample was run in triplicate. Data were collected and displayed in one-dimensional histograms comprising 5 log decades and dot-plot cytograms based on a combination of light scatter signals (FS *vs* SS) and light scatter signals *vs* fluorescence (FS *vs* FL4; SS *vs* FL4; FL1 *vs* FL4). Data analysis was performed on the EC800 software 1.3.6. version (Sony Biotechnology Inc., USA).

Flow cytometric analysis using viability staining dyes (SYTO-BC and PI) was applied to quantitatively assess the effect of the three biocides tested – triclosan, CTAB, and glutaraldehyde on *N. amarae* and *S. natans*. To assess the percentage of live bacteria (stained green), injured bacteria but not dead (stained green and red), and dead bacteria (stained red) upon biocide treatment, only the lowest and the highest concentration of each biocide was assayed. Nevertheless, only the highest concentration of each biocide that showed significant results was assayed at 72 h. To figure out whether the injured bacteria population is fated to die or is able to recover after removal of the highest concentration of biocide, a recovery assay was performed. To carry on the assay, two cultures (flask 1 and flask 2) were grown simultaneously. After 24 h of exposure to the biocide, a sample was taken from flask 1 to be analysed by flow cytometry. The remaining culture was left growing to 72 h. After 24 h of exposure to the biocide, the medium was

removed from flask 2 and the culture was left growing in fresh medium to 72 h as a follow-up for the recovery assay. Two other cultures were used as control (untreated) and control with DMSO (≤ 2.5 %).

Epifluorescence microscopy procedure

Cell viability assessment is crucial for the evaluation of cell health when exposed to toxic compounds, and is usually analysed with the help of several parameters (e.g. integrity of cell membrane) (Rampersad 2002). Along with the flow cytometry, epifluorescence microscopy was performed using SYTO-BC and PI, as viability staining dyes., to visualize the effect of the three biocides studied on the morphology of filamentous bacteria (namely, *N. amarae* and *S. natans*.)

Samples were taken and fluorescence microscope images of viable cells (live) stained with SYTO-BC (green) and non-viable cells (dead) stained with PI (red) were obtained. Samples for the two strains were taken after 24 h and 72 h of biocide treatment at the highest concentration (TH, CH, GH), as well as in the 72 h recovery assay.

The bacterial cells were incubated in the dark for 10 minutes and analysed with an epifluorescence microscope BX51 (Olympus, Japan) using fluorescein isothiocyanate (FITC) (*excitation* spectra (EX) 470 – EM BP490/520) and tetramethylrhodamine (TRITC) (EX 530 – EM BP550/590) filters to visualize live and dead cells respectively. All images were taken using *Citifluor* non-fluorescent immersion oil (Agar scientific, UK) containing antifadent AF87, designed for fluorescent microscopy (high magnifications) to reduce the fading of fluorescence dyes used for labelling biological species. For all parameters tested, multiple images were captured with an DP72 digital camera (Olympus, Japan). Each image was divided into four compartments using a grid system from the Olympus CellSens standard software 1.16 version. The number of live and dead cells in each compartment was manually counted and averaged. The percentage of live and dead cells was estimated. Moreover, the size of at least 50 filaments was averaged after selecting random compartments of each image collected from the epifluorescence microscope.

Apart from medium negative control, unstained culture control, SYTO-BC and PI double stained control and SYTO-BC and PI double stained DMSO control were observed under epifluorescence microscopy. Only the results for double stained culture are shown. Both strains showed no autofluorescence.

4.2.4 Statistical analysis

Statistical data analysis, comparisons and graphics were performed in Microsoft Excel (USA) and GraphPad Prism software 5.0 version (USA). The one-way and two-way analysis of variance (ANOVA) were performed using GraphPad Prism. Cell viability was expressed as mean ± standard error mean (SEM, n = 3). The normal distribution of data for the parametric test ANOVA was confirmed using the Shapiro-Wilk test and the non-normally distributed data were analysed using the non-parametric test Kruskal-Wallis. Post-hoc tests were used to confirm significant differences between groups. Tukey´s multiple comparison test and Dunn´s multiple comparison tests were carried out for the parametric and non-parametric tests, respectively. Bonferroni multiple comparison test was also carried out. Statistically significant differences were determined for *p*-values ≤ 0.05(*), 0.001(**), and 0.0001(***) for one-way ANOVA and *p*-values ≤ 0.05(*), and 0.01(**), 0.001 (***) for two-way ANOVA.

4.3 Results and discussion

4.3.1 XTT reduction assay

As explained above, a range of triclosan concentrations was adjusted to obtain the lowest (10 μg/mL) to the highest (200 μg/mL) biocidal effect, in a single end-point assay (24 h) (Figure 4.3 (a)). Within this range, the viability of *N. amarae* decreased with increasing concentrations, in a dose dependent manner (LC_{50} = 25 μg/mL; 86 μM). The slight stimulation caused by the biocide at the lowest concentration is statistically non-significant as well as the inhibition caused by 20 μg/mL, tough the LC_{50} is close to this. Statistically significant different values were found between the control (0 μg/mL) and biocide concentrations of 40 μg/mL ($p \leq 0.001$) and above ($p \leq 0.0001$). At the highest biocide concentration (200 μg/mL) a percentage of 86 % of the culture was non-viable.

The viability of *N. amarae* decreased with increasing concentrations of CTAB, also in a dose-dependent manner (Figure 4.3 (b)). CTAB was the most effective biocidal agent against *N. amarae* (LC_{50} = 3 μg/mL; 8 μM) and approximately 4 % of bacteria were viable after 24 h of incubation at 40 μg/mL. Statistically significant differences ($p \leq 0.05$) were observed from the lowest concentration tested (1 μg/mL). At the highest concentration (40 μg/mL) a percentage of 96 % of the culture was non-viable.

Finally, the viability of *N. amarae* decreased with increasing concentrations of glutaraldehyde, in a dose-dependent manner (Figure 4.3 (c)). Based on the LC_{50}, among the three biocides tested, glutaraldehyde

was the less effective biocidal agent (LC_{50} = 517 µg/mL; 5164 µM) and statistically significant differences between control *vs* biocide concentration were found after a treatment of 400 µg/mL ($p \leq 0.001$); 800 µg/mL ($p \leq 0.0001$) and 1000 µg/mL ($p \leq 0.0001$). After a treatment of 800 µg/mL to 1000 µg/mL more than 85 % of the culture was non-viable.

Overall, all biocides tested showed a dose-dependent biocidal effect against *N. amarae*. Different range of lethal concentrations were obtained for all biocides, indicating that CTAB was the most effective biocide agent in 24 h, followed by triclosan, and glutaraldehyde. On the other hand, by comparing the percentage of viability between two consecutive concentrations surrounding the LC_{50} (20 to 40 µg/mL; 2 to 4 µg/mL; and 400 to 800 µg/mL for triclosan, CTAB and glutaraldehyde, respectively) the biocidal relative increase was more pronounced for glutaraldehyde > CTAB > triclosan. In fact, the decrease in the viability between two consecutive concentrations of biocide was of 5-fold for glutaraldehyde, 4-fold for CTAB, and 2-fold for triclosan.

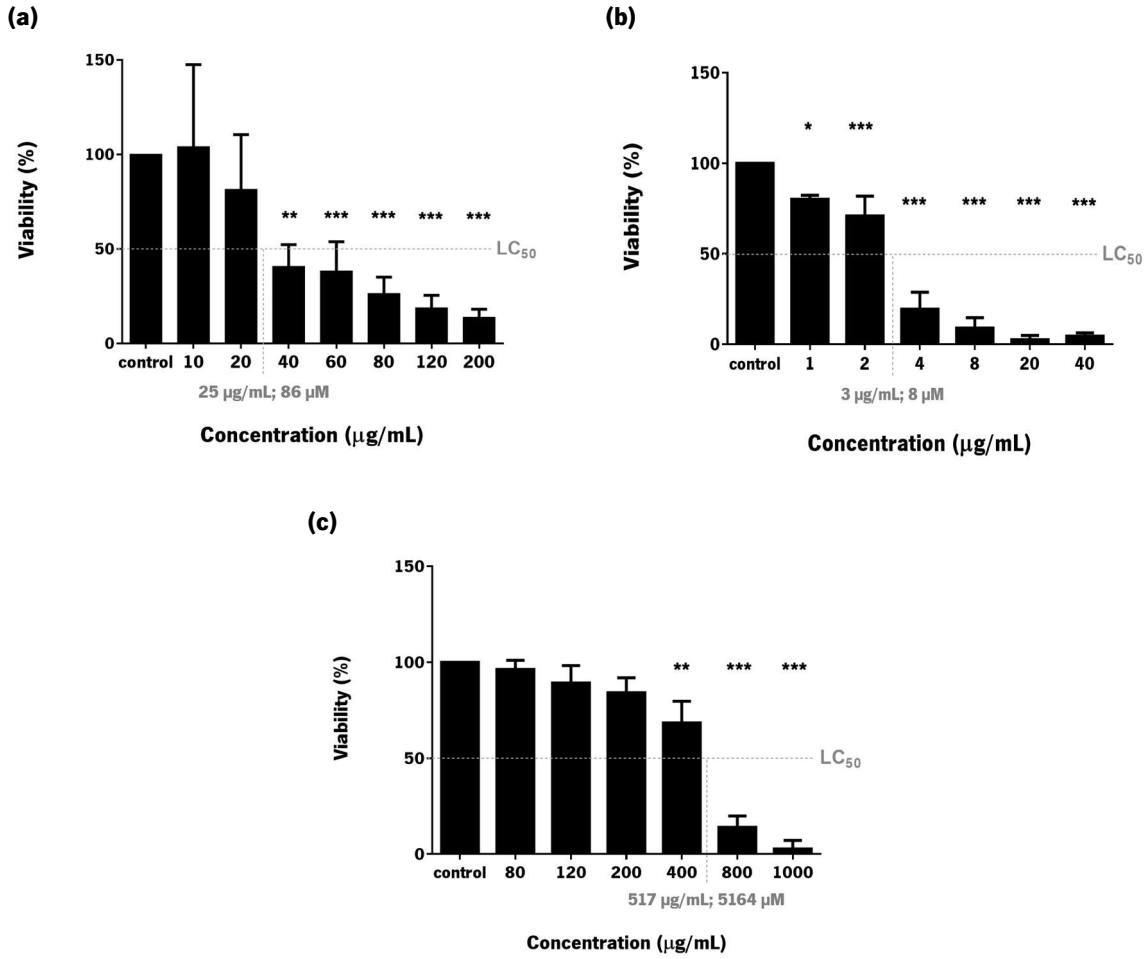

Figure 4.3: Viability (%) of *N. amarae* when exposed to **(a)** triclosan, **(b)** CTAB and **(c)** glutaraldehyde concentrations (µg/mL) in the single end-point assay (24 h). The LC_{50} value of each biocide after 24 h is represented by a dashed line. All the columns show the mean ± SEM (n=3). Statistically significant difference with control (0 µg/mL): * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

The viability of *S. natans* decreased with increasing concentrations of triclosan, in a dose dependent manner (LC_{50} = 0.4 μg/mL; 1 μM) (Figure 4.4 (a)). Statistically significant different values were found between the control (0 μg/mL) and biocide concentrations of 0.25 μg/mL ($p \leq 0.05$) and above ($p \leq 0.0001$). After a treatment of ≥ 2 μg/mL, less than 10 % of bacterial cells remained viable. At the highest concentration (8 μg/mL) all bacteria were non-viable.

In the case of CTAB, the viability of *S. natans* also decreased with increasing concentrations of this biocide, in a dose-dependent manner (LC_{50} = 3 μg/mL; 8 μM) (Figure 4.4 (b)). Statistically significant different ($p \leq 0.0001$) were found at concentrations of 4 to 30 μg/mL. After a treatment of ≥ 8 μg/mL, less than 10 % of bacterial cells remained viable. At the highest concentration (30 μg/mL), 97 % of the culture was non-viable.

Finally, the viability of *S. natans* decreased with increasing concentrations of glutaraldehyde, in a dose-dependent manner for concentrations ≤ 80 μg/mL (Figure 4.4 (c)). Unexpectedly, at higher concentrations (120 μg/mL and 200 μg/mL), the decreasing viability trend in *S. natans* appeared to reverse from 30 % to 44 %. This can be due to a different mechanism of death at concentrations above a certain point: in fact, death by apoptosis can result in interference with XTT results by the reactive-oxygen species involved in the process of apoptosis. Therefore, due to this fact, LC_{50} was calculated taking into account the exclusion of values above 80 μg/mL, obtaining an LC_{50} = 75 μg/mL; 749 μM.

Overall, all biocides showed a dose-dependent effect on *S. natans* viability. Different range of lethal concentrations were found for all biocides, indicating that triclosan was the most effective biocidal agent, followed by CTAB, and glutaraldehyde. Moreover, considering the percentage of viability between the two consecutive concentrations surrounding the LC_{50} (0.25 to 0.5 μg/mL; 2 to 4 μg/mL; and 75 to 80 μg/mL for triclosan, CTAB and glutaraldehyde, respectively), the increase in the effect was more pronounced for CTAB > glutaraldehyde and triclosan. The decrease in the viability percentage between two consecutive concentrations of biocide was of 3-fold for CTAB, and 2-fold for glutaraldehyde and triclosan.

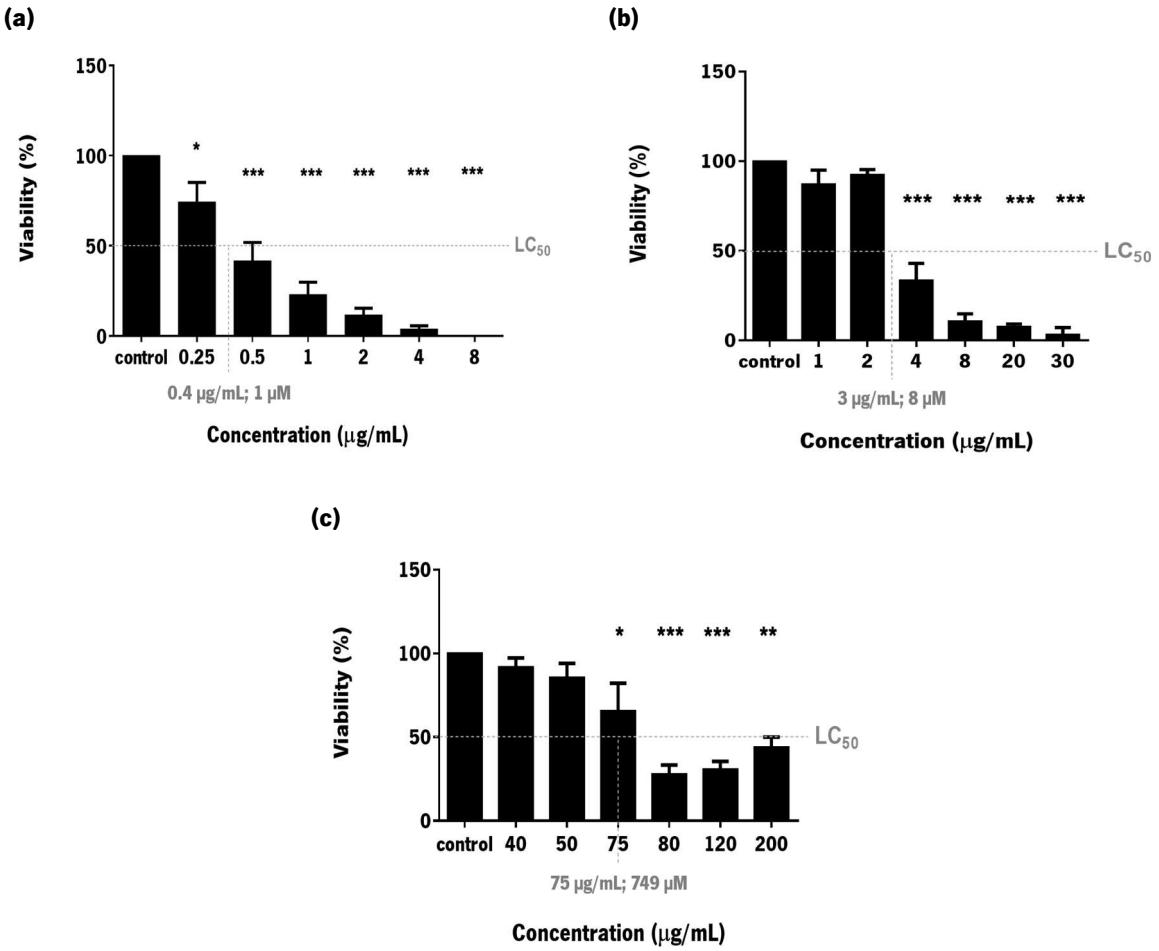

Figure 4.4: Viability (%) of *S. natans* when exposed to **(a)** triclosan, **(b)** CTAB and **(c)** glutaraldehyde concentrations (µg/mL) in the single end-point assay (24 h). The LC_{50} value of each biocide after 24 h is represented by a dashed line. All the columns show the mean ± SEM (n=3). All the columns show the mean ± SEM (n=3). Statistically significant difference with control (0 µg/mL): * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

As mentioned before, susceptibility to biocides varies among Gram positive and negative bacteria, being Gram negative bacteria reported to be generally less susceptible to biocides (Wales & Davies 2015). This difference is thought to be due to the fact that the Gram negative bacteria may have a high cell impermeability, so that the access of the biocide to its target sites can be reduced, causing the cells intrinsically less susceptible to biocides (Johnson *et al.* 2002). Some studies reported inhibition of Gram positive *Staphylococcus* at concentrations as low as 0.06 µg/mL of triclosan (Slater-Radosti *et al.* 2001). On the other hand, most strains of Gram negative *Pseudomonas aeruginosa* require concentrations varying between 100 to 1000 µg/mL of triclosan (Fraise *et al.* 2013). Paradoxically, in our study the Gram positive *N. amarae* was clearly less susceptible than the Gram negative *S. natans* showing a 25-fold difference between them. Due to the use of a single species from each Gram type, it was not possible to establish a correlation between the action mechanism of triclosan with the composition type of the

cellular membrane, but the layer of peptidoglycan of *N. amarae* can explain an increased difficulty to enter the cell and affect the lipid synthesis.

The action mechanism of the cationic surfactant CTAB relies on the ability to induce cell lysis by adsorption on cell wall/cytoplasmic membrane (Ludensky 2005; Guo *et al.* 2012; Abreu *et al.* 2013). CTAB is an effective biocidal agent against bacteria. Its activity was previously reported against filamentous bacteria from activated-sludge due to its enhanced ability to induce cell lysis (Bitton 2005; Bratby 2016; Seka *et al.* 2001; Yang *et al.* 2013). It is known that other surfactants such sodium dodecyl sulfate (SDS) and Triton X-100 can cause the collapse of the phospholipid bilayer that surrounds cell surface in Gram negative filamentous bacteria. Caravelli *et al.* (2007) reported that cell lysis was induced by Triton X-100, when the Gram negative *S. natans* was exposed to a concentration of 120 µg/mL. More recently, Guo *et al.* (2012) described the effect of CTAB on filamentous bulking sludge caused by chlorine resistant bacteria Type 021N (Gram negative), reporting that the cationic surfactant CTAB may have a stronger penetration capacity through the cell wall (Zhang *et al.* 2015). In this study, it was not possible to establish a correlation between the action mechanism of CTAB and the composition type of the cellular membrane, since the range of toxicity for both bacteria strains were similar concentrations (1 to 40 µg/mL for *N. amarae* and 1 to 30 µg/mL for *S. natans*) and the LC_{50} is exactly the same (3 µg/mL; 8 µM). So, the layer of peptidoglycan in the Gram positive *N. amarae* in one side or the double membrane system of Gram negative *S. natans* in the other, can result in equal result on the resistance to CTAB. On the other hand, among the three biocides, CTAB was the most effective biocidal agent in *N. amarae* cultures.

Glutaraldehyde has a broad range of activity against bacteria, fungi, and viruses and a high biocidal effectiveness, especially with persistent organisms, due to its good penetration ability (Miao *et al.* 2019). Glutaraldehyde is capable to interact with constituents of the wall and cellular membrane of the microorganisms (Pereira 2001; Pereira & Vieira 2001). Yoo (2018) reported that glutaraldehyde is an effective biocidal agent against *Mycobacteria* in general, but it is not very effective in killing some non-tuberculous *Mycobacteria* (e.g., *M. chelonae, M. xenopi,* or *M. massiliense*). Other authors described a strong binding of glutaraldehyde to outer proteinaceous layers of organisms such as *E. coli* (Gram negative) and *Staphylococcus aureus* (Gram positive) (McDonnell & Russell 1999). Once again, in the present study, the Gram positive bacteria *N. amarae* was clearly less susceptible than the Gram negative *S. natans* showing a 5-fold difference. Due to the use of a single species from each Gram type, it was not possible to establish a correlation between the action mechanism of glutaraldehyde with the composition

of the cellular membrane, but the existence of a thick external peptidoglycan layer can account for the increase difficulty to access the proteins of the cell wall.

Comparing both strains, *N. amarae* showed to be less susceptible to triclosan and glutaraldehyde than *S. natans*, although *N. amarae* is Gram positive and *S. natans* is Gram negative, contradicting, as said above, to what is reported by Johnson *et al.* (2002) and Wales & Davies (2015). It must be noted that these authors do not refer the present biocides specifically. The LC_{50} obtained also corroborate these observations. CTAB had a similar effect on both strains on what dose range and LC_{50} is respectively equal. Nevertheless, CTAB was the most toxic compound among the three used to *N. amarae* and the intermediate toxic when considering the assays with *S. natans*. Triclosan was the most toxic compound to *S. natans*.

4.3.2 Live/dead viability assay (Cytometry and Epifluorescence microscopy)

4.3.2.1 Cytometry

Concentrations of biocides used in this assay were the same used in the XTT assay for each bacterial strain.

A stacked graph representing the average percentage of live, injured and dead cells for *N. amarae* is displayed in Figure 4.5. A code composed of two letters was attributed for the different concentrations of biocides (T for triclosan, C for CTAB, and G for glutaraldehyde). The letter (L) was used for the lowest concentration, and the letter (H) for the highest concentration of biocide. The (R) letter was added to identify in the graph the recovery assay performed in 72 h (48 h after de biocide removal).

The effect of the lowest biocide concentration (10 µg/mL for triclosan – TL; 1 µg/mL for CTAB – CL and 80 µg/mL for glutaraldehyde – GL) *vs* the highest concentration (200 µg/mL for triclosan – TH; 40 µg/mL for CTAB – CH and 1000 µg/mL for glutaraldehyde – GH) was assessed for each end-point of 24 h (Figure 4.5 (a)) and 72 h (Figure 4.5 (b)).

The 24 h untreated culture of *N. amarae* showed that 94 % of population was viable (live population), with a slight percentage of injured cells (4 %). The remaining 2 % were dead bacteria. The viability of cells treated with DMSO was not significantly affected (85 % viable cells), thereby ruling out the occurrence of solvent-induced cytotoxicity.

Regarding the effect of each biocide after 24 h of treatment, the percentage of the live population of *N. amarae* decreased with the increase of biocide concentration, as expected.

For triclosan, the live population was reduced from 82 % at TL to 13 % at TH, at 24 h, dead population increased from 8 % (TL) to 24 % (TH), the injured bacteria also increased (TL= 10 %; TH = 63 %). A total of 87 % of the culture was injured or dead after a treatment of triclosan at a concentration of 200 µg/mL.

The live population treated with CTAB was reduced from 62 % at CL to 2 % at CH. The dead population increased from 23 % (CL) to 51 % (CH), the remaining bacteria were injured (CL= 15 %; CH = 47 %). A total of 98 % of the culture was injured or dead after a treatment of CTAB at a concentration of 40 µg/mL.

Surprisingly, no significant differences were found in live (GL = 75 %, GH = 76 %), dead (GL = 7 %; GH = 4 %) and injured (GL = 18 %; GH = 20 %) populations after the treatment with glutaraldehyde between both concentrations (80 µg/mL and 1000 µg/mL). Nevertheless, this was a transitory response and could be due to the glutaraldehyde properties of cell fixation (Hu *et al.* 2017), preventing the entrance of PI through the cell wall or a delay in the toxicity action of glutaraldehyde. In fact, after this period, toxicity has showed to increase significantly, as next described, even in the case of the biocide removal.

After 72 h of culture the untreated *N. amarae* showed a significant ($p \leq 0.001$) reduction of the viable population, comparing with the 24 h time of incubation. Regarding the effect of each biocide after 72 h of treatment, the percentage of the live population of *N. amarae* increased slightly but not significantly for triclosan and CTAB. On the contrary, a significant decrease of live bacteria was observed when treated with glutaraldehyde ($p \leq 0.05$). In what mortality and injuries concerns, there were no significant changes in the case of triclosan but, in the case of CTAB, the dead cells decreased but the percentage of injured cells increase significantly, thus leading to subsequent death. Glutaraldehyde causes increased injury in 72 h and maintained the dead cells percentage.

Estimation of live, injured, and dead bacteria was performed for bacteria cultured for the recovery assay. Comparing the same biocide concentrations (TH and TR), the results suggest that the bacteria were not able to recover from the triclosan treatment in the next 48 h after the removal of the biocide. In the graph, the percentages of live, injured and dead bacteria were similar considering the cultures treated with triclosan and the recovered cultures and were also not significantly different from those of the 24 h assay.

For the treatment with CTAB, the percentage of live bacteria in the recovered culture increased slightly but not significantly and the scenario is statistically similar to that of the treated population in terms of dead, injured and live cells.

In the case of glutaraldehyde, regardless the increase of live bacteria observed in the recovered cultures and the decrease in injured cells, the statistical analysis did not show significant differences between 72 h treated cells and those of the recovered cultures (p>0.05).

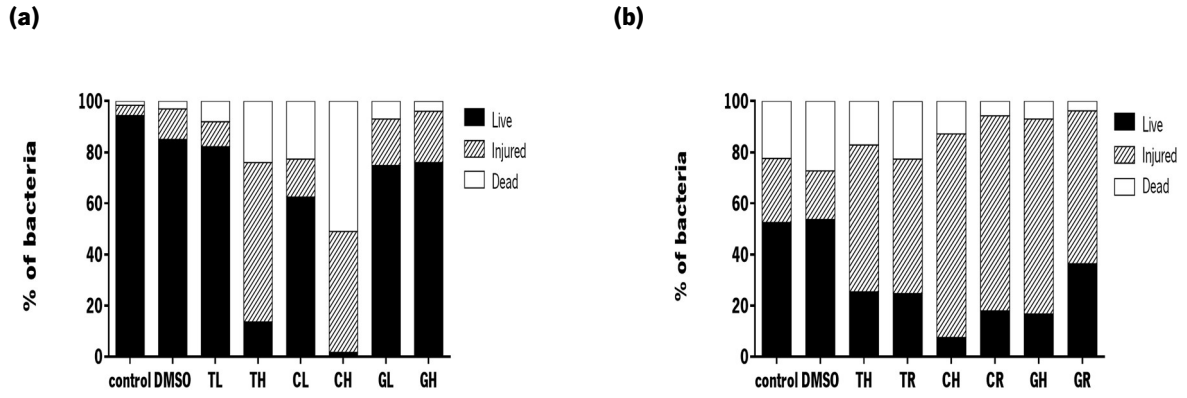

Figure 4.5: Average (%) of live, injured and dead cells for *N. amarae* present in the controls (0 µg/mL and DMSO) and in the lowest and highest concentrations (µg/mL) of the three biocides studied (triclosan (TL, TH, TR), CTAB (CL, CH, CR) and glutaraldehyde (GL, GH, GR) respectively) after **(a)** 24 h, **(b)** 72 h of biocide treatment and after the recovery (R) assay at 72 h for the highest concentration tested. All the columns show the mean (n=3).

Summarising, cells could not significantly recover from the biocidal action in 48 h after the biocide removal in any case. Overall, among all biocides tested and considering this evaluation, CTAB was the most effective on *N. amarae*, followed by triclosan and glutaraldehyde, if the effects were to be considered individually at each time of sampling and assessment. So, the ranking of the biocidal capacity was maintained in this assay: CTAB > triclosan > glutaraldehyde, as in the XXT assays. Nonetheless, the effect of triclosan seems to be more rapid as it is maintained along the entire period of time since the 24 h sampling, considering dead, injured and live cells; the effect of CTAB is more pronounced if the sum of dead cells + injured cells is to be considered and the effect of glutaraldehyde was not significant after 24 h exposure but its action increased with time, even if the biocide was removed, which is an interesting result.

A stacked graph representing the average percentage of live, injured and dead cells for *S. natans* is displayed in Figure 4.6. The effect of the lowest biocide concentration (0.25 µg/mL for triclosan – TL; 1 µg/mL for CTAB – CL and 40 µg/mL for glutaraldehyde – GL) *vs* the highest concentration (8 µg/mL for

triclosan – TH; 30 µg/mL for CTAB – CH, and 200 µg/mL for glutaraldehyde – GH) was assessed for each end-point of 24 h (Figure 4.6 (a)) and 72 h (Figure 4.6 (b)).

The 24 h untreated culture of *S. natans* showed that 86 % of population was viable (live population), with a small percentage of dead cells (7 %). The remaining 7 % were injured cells. The viability of cells treated with DMSO was not significantly affected (84 % of viable cells), thereby ruling out the occurrence of solvent-induced cytotoxicity. Regarding the effect of each biocide after 24 h of treatment, the percentage of the live population of *S. natans* decreased with the increase of biocide concentration in all cases, as expected. For triclosan, the live population was reduced from 78 % at TL to 62 % at TH. The dead population increased from 13 % (TL) to 25 % (TH), and the remaining bacteria were injured (TL = 9 % and TH = 13 %). A total of 38 % of the culture was injured or dead after a treatment of triclosan at a concentration of 8 µg/mL.

The live population treated with CTAB was reduced from 89 % at CL to 3 % at CH, the dead population increased from 5 % (CL) to 61 % (CH), the remaining injured bacteria also increased (CL = 6% to CH = 36 %). A total of 97 % of the culture was injured or dead after a treatment of CTAB at a concentration of 30 µg/mL.

No significant differences were found in live (75 %; 57 %), injured (11 %; 15 %) and dead (14 %; 28 %) populations after the treatment of 24 h with glutaraldehyde at both concentrations (40 µg/mL and 200 µg/mL), although there were increased non-significant increases of dead cells and increased non-significant of injured cells from the lowest to the highest concentrations.

No significant differences were found between different times of incubation for the untreated *S. natans*. Regarding the effect of each biocide after 72 h of treatment, the percentage of the live population decreased significantly ($p \leq 0.0001$) with triclosan treatment.

No significant differences were found between the two times of incubation for CTAB and glutaraldehyde treatment, but one must note that the number of live cells in the case of CTAB, after 24 hours was already very low, so it is possible to say that the highest concentration maintained the biocidal effect on the culture, unless the biocide was removed.

The percentage of dead population increased with the permanence of the biocide in the next 72 h for all biocides treatments but this increase was only significant for triclosan and CTAB, in this latter case, from injured to dead cells.

Estimation of live, injured, and dead bacteria was performed for bacteria cultured for the recovery assay. Regardless the increase of live bacteria observed in the recovered cultures the statistical analysis did not show significant differences in the next 48 h after removal of the biocides (p>0.05).

Overall, among all biocides tested and considering this methodology, CTAB was the most effective on *S. natans* in 24 h, followed by triclosan and then, glutaraldehyde, the same order obtained in the assays with *N. amarae*. Here too the higher sensitivity of the Gram negative *S. natans* is apparent as it occurred in the XTT assays.

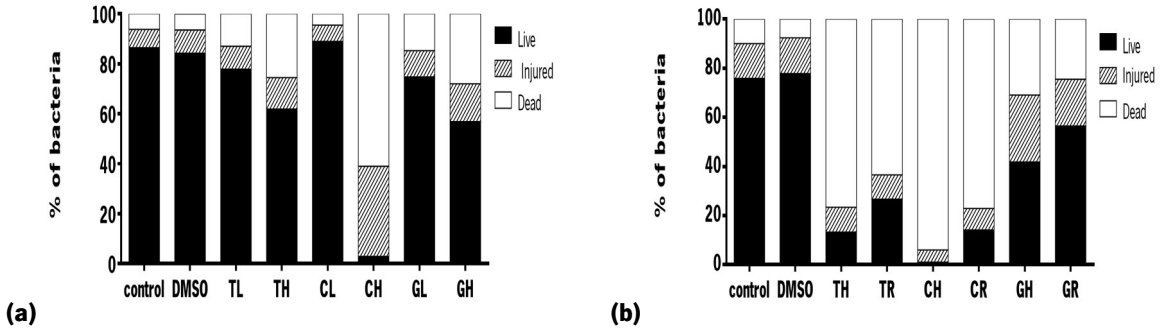

Figure 4.6: Average (%) of live, injured and dead cells for *S. natans* in the controls (0 µg/mL and DMSO) and in the lowest and highest concentrations (µg/mL) of the three biocides studied (triclosan (TL, TH, TR), CTAB (CL, CH, CR) and glutaraldehyde (GL, GH, GR) respectively) after **(a)** 24 h, **(b)** 72 h of biocide treatment and after the recovery (R) assay at 72 h for the highest concentration tested. All the columns show the mean (n=3).

In Supplementary Materials are displayed the mean values, the standard deviation (SD) and the standard error mean (SEM) of controls and biocides concentrations after 24 h, 72 h of biocide treatment and after recovery assay at 72 h for *N. amarae* and *S. natans* (live, injured and dead cells), in Table 4.13 and Table 4.14, respectively. In addition, the supplementary materials also display box and whiskers plots used to compare the effect of the highest concentration of biocides on live bacteria *vs* time of treatment for *N. amarae* (Figure 4.10) and *S. natans* (Figure 4.11).

Summarising, it was shown that the biocides had significant effects on death, injury and recovery in the two strains but in different ways: for instance, in *N. amarae*, triclosan had a significant higher biocidal effect in the highest concentration and removal of the biocide does not alter the scenario (a slight recovery although not significantly in the period of 72 h), CTAB also had a higher biocidal effect in the highest concentration but the recovery seems to be more promising if the biocide was removed (although also not significantly in the period of 72 h) and finally, glutaraldehyde had similar effects (by this assessment methodology) in 24 h but the biocidal effect increased in 72 h even if the biocide was removed. All these

differences with time and with biocidal removal, corroborate the hypothesis of different biocidal mechanisms taking place.

Considering *S. natans*, it is obvious that this strain is clearly more susceptible than *N. amarae* and one of the interesting aspects is that only a slight percentage of the counting corresponds to injured cells, meaning that the biocidal effect is also faster. CTAB was also the most toxic among the three biocides, in the case of this assay, and its action followed a pattern very similar to that of *N. amarae*. Glutaraldehyde also acted in a similar way, although with a slight better indication in the recovery of the cells, tough also not significantly in 48 h. Triclosan caused a different response as in the case of *S. natans,* the action was also prolonged in time but the removal of the biocide also enabled a faster recovery of the cells although also non-significant.

4.3.2.2 Epifluorescence microscopy

The presented results correspond to 24 h and 72 h of biocide treatment at the highest tested concentration, including the recovery assay. In Figure 4.7, the control culture of *N. amarae* (0 μg/mL) after 24 h and 72 h of treatment, showed that most bacteria were live and viable (green). The bacteria displayed filaments and in almost all fields it was possible to observe small aggregates (flocs). A few non-viable cells (red) were observed at 24 and 72 h, as expected.

The results for *N. amarae* after 24 h of treatment with triclosan clearly showed lower intensity of the green fluorescence (13% of viable cells) and a fragmentation of the filaments to single cells, when compared to the control. After 72 h of treatment, a low percentage of bacteria remained viable, although higher than the previous (25 %), and almost no filaments were observable.

After 24 h of treatment with CTAB most bacteria were non-viable (98 %). However, no changes in the morphology were observed, since the bacteria remained filamentous. Morphological changes were only detectable at 72 h (fragmentation) and 8 % of cells were now viable.

After 24 h of treatment with glutaraldehyde, most bacteria remained viable (76 %). However, some filament fragmentation was visualized. Most bacteria were in single cells after 72 h of treatment and a significant decrease in viability was observed (17%).

Regarding the recovery assay with triclosan, the bacteria remained as non-viable single cells and not able to produce new filaments and the percentage of viable cells was near that of the exposed culture. Cultures

in the recovery assay with CTAB and glutaraldehyde seemed to be on the recovery process, with a higher number of viable green bacteria comparing with treated bacteria. Nevertheless, no new filaments were observable and the bacteria remained as single cells in the 48 h following the biocide removal. The ability of producing flocs was lost in this period of time.

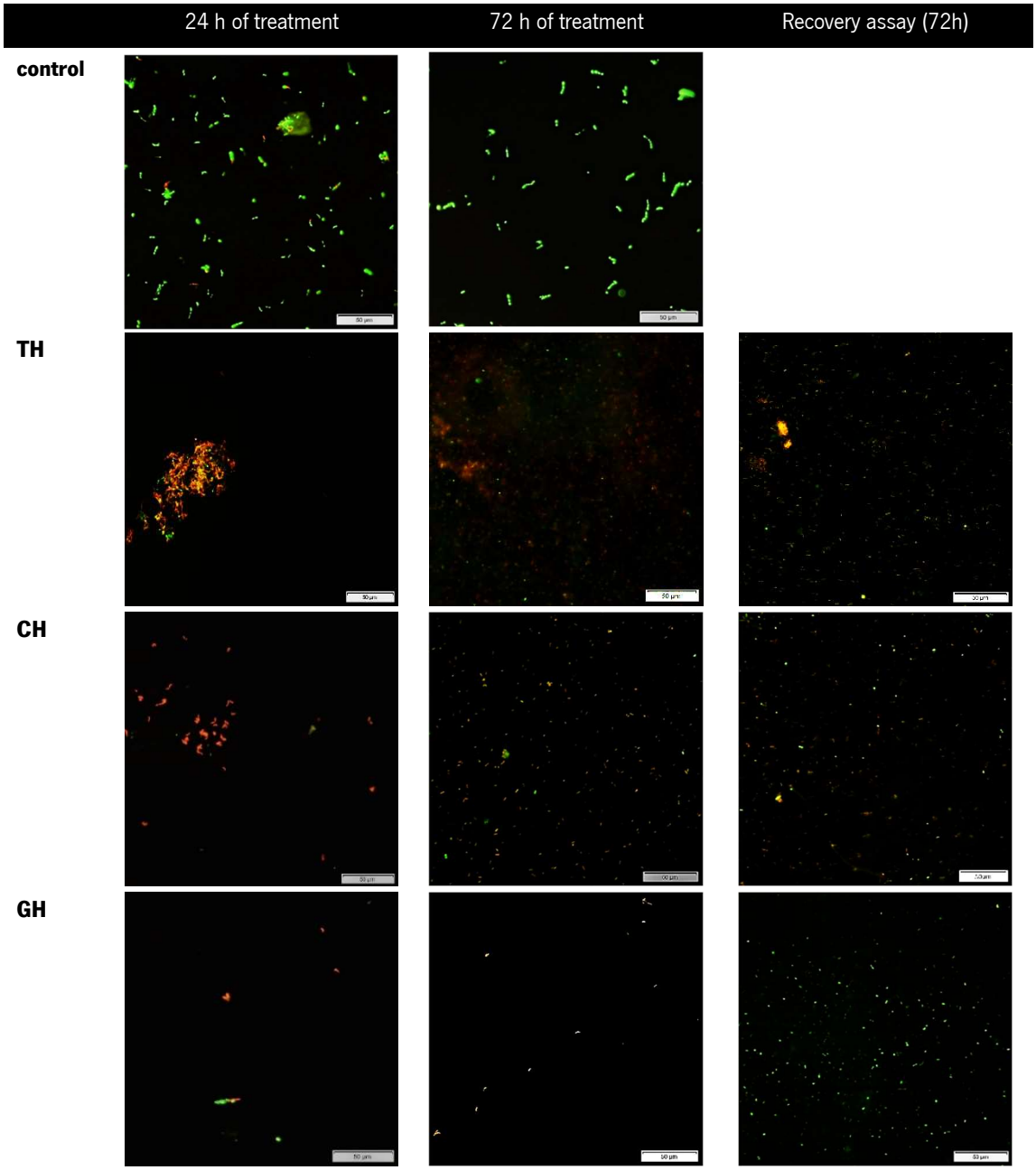

Figure 4.7: Epifluorescences images of *N. amarae* in the control (0 µg/mL) and in the highest concentration of triclosan (TH), CTAB (CH) and glutaraldehyde (GH) after 24 h, 72 h of biocide treatment and after the recovery assay at 72 h. Live/dead cell viability assay showing live cells stained with SYTO-BC (green) and dead cells with PI (red), with the amplification of 20 X (scale bars = 50 µm).

In Figure 4.8, the control culture of *S. natans* (0 µg/mL) after 24 h and 72 h of the higher dose treatment, showed that most bacterial cells were viable live (green). The morphology of the bacteria was filamentous and in almost all fields it was possible to observe small aggregates (flocs). A few non-viable cells (red) were observed at 24 and 72 h, as expected.

The results for *S. natans* after 24 h of treatment with triclosan clearly showed lower intensity of the green fluorescence and fragmentation of the filaments to single cells, when compared to the control. A percentage of 62 % of the bacterial culture remained viable. However, after 72 h of treatment, the percentage of viable cells decreased to 13 % and they remained as non-filament.

After 24 h of treatment with CTAB most bacteria were already non-viable (97 %). In contrast to *N. amarae*, CTAB immediately affected the capacity of filamentation as almost no filaments could be seen. After 72 h of treatment about 99 % of population was non-viable.

After 24 h of treatment with glutaraldehyde most bacteria remained viable (57 %). However, changes in the morphology were observed and some fragmentation of the filament was visualized. Most bacteria were found in single cell morphology after 72 h of treatment being only 42 % viable.

Regarding to the recovery assay, the cells seemed to be recovering, with a higher number of viable green bacteria comparing with treated bacteria, in all three cases. Nevertheless, no new filaments were observable and the bacteria remained as single cells. The capability of producing flocs was lost.

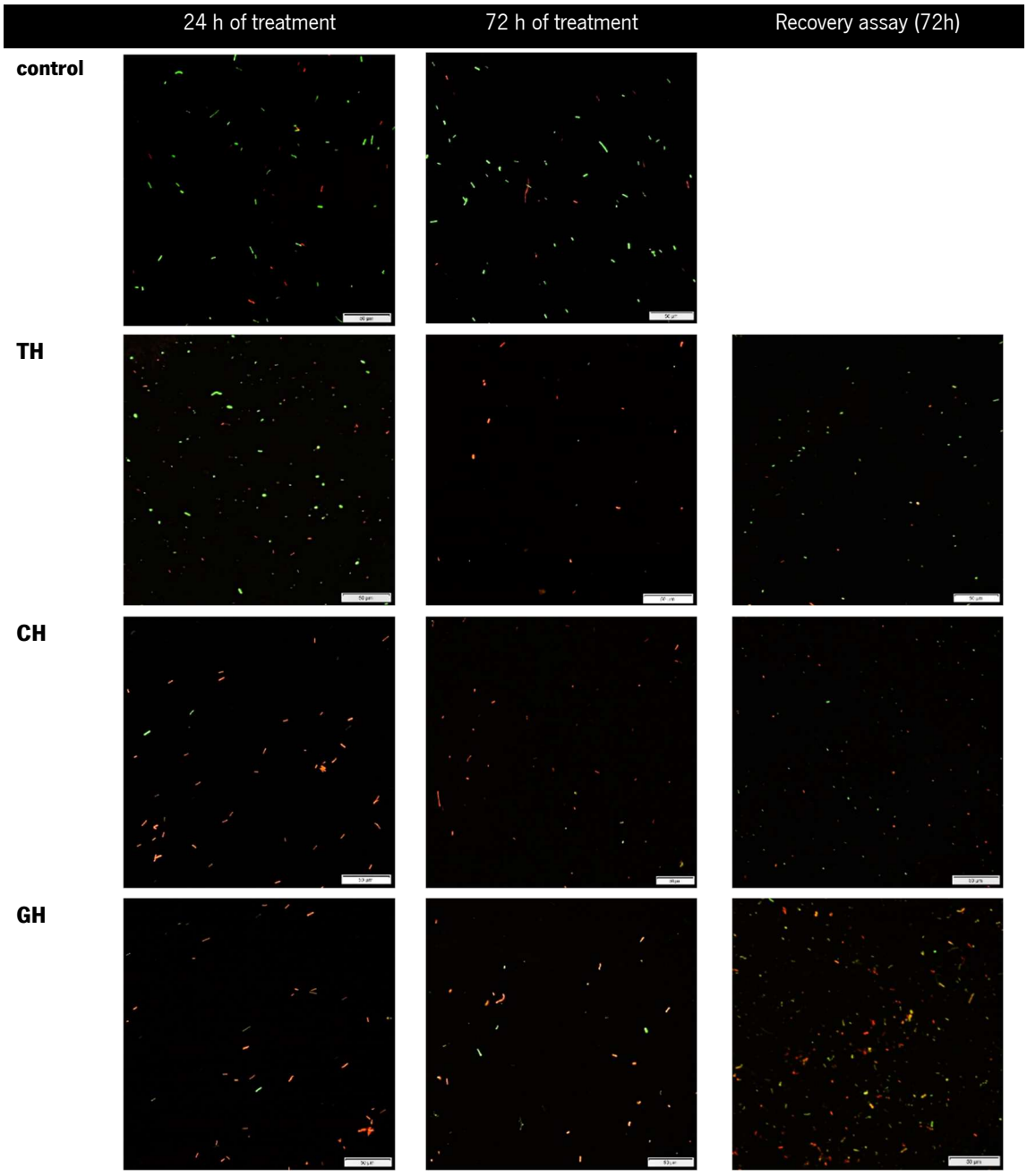

Figure 4.8: Epifluorescences images of *S. natans* in the control (0 µg/mL) and in the highest concentration of triclosan (TH), CTAB (CH) and glutaraldehyde (GH) after 24 h, 72 h of biocide treatment and after the recovery assay at 72 h. Live/dead cell viability assay showing live cells stained with SYTO-BC (green) and dead cells with PI (red), with the amplification of 20 X (scale bars = 50 µm).

The results obtained by microscopy allow to conclude that the main effects of the tested biocides are on the filamentation and on viability. To confirm the effect on filamentation, the size of the filaments, in untreated (control) and after 24 h of treatment with biocides, was recorded and the average size and standard deviation is presented in Figure 4.9. After 24 h of treatment, *N. amarae* (Figure 4.9 (a)) showed a significant reduction of the filament size when comparing the control (0 µg/mL) in the cultures treated with triclosan ($p \leq 0.001$) and glutaraldehyde ($p \leq 0.0001$). For both biocides, one of the effects was the

fragmentation of filaments. CTAB did not affect the size of the filaments as compared with the control (p>0.05), even when cells were non-viable (red), meaning that even considering the potential action on the cell walls and membranes (as a surfactant), filaments maintained its continuity and structure until, at least, 24 h.

After 24 h of treatment, *S. natans* (Figure 4.9 (b)) showed significant different filament size when comparing the control (0 µg/mL) with cultures treated with all biocides. Although size reduction was visually and statistically ($p \leq 0.0001$) noticeable in all treated cultures, some biocides showed a more pronounced effect than others. Triclosan was the biocide with the major effect in filament size.

These results are of utmost importance as filaments of *N. amarae* will probably maintain its action as bulking agents for at least 24 h, in the case of CTAB adding. It is not expected to have such an occurrence if *S. natans* is the bulking agent. Due to the use of a single species from each Gram type, it is not possible to infer that this was due to the composition of the wall.

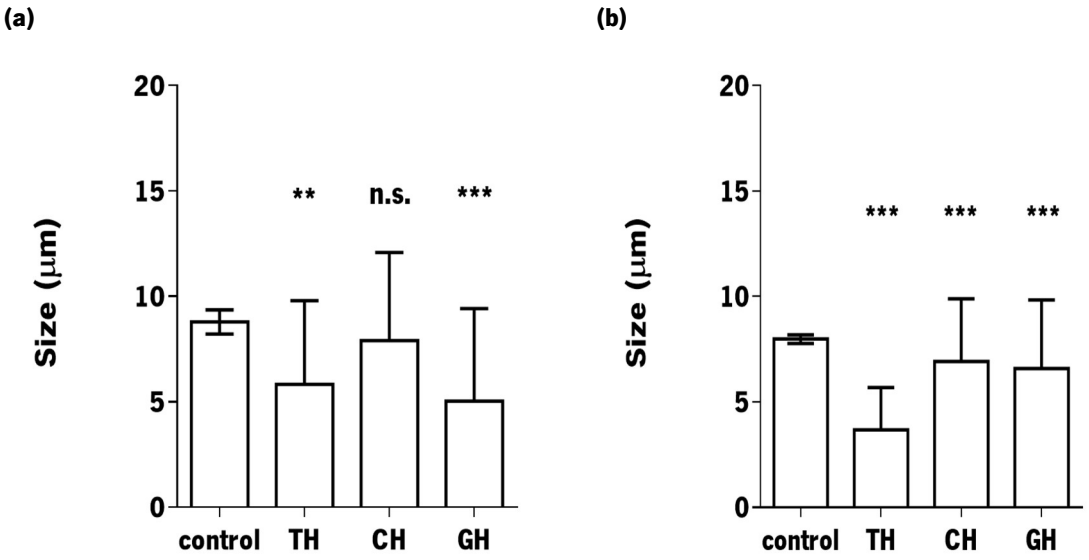

Figure 4.9: Average filament size (µm) of **(a)** *N. amarae and* **(b)** *S. natans* cells present in the control (0 µg/mL) and in the highest concentration (µg/mL) of the three biocides studied (triclosan (TH), CTAB (CH) and glutaraldehyde (GH) respectively) after 24 h. All the columns show the mean ± SEM (n=3). n.s. Not statistically significant difference with the control (0 µg/mL); Statistically significant difference with control (0 µg/mL): ** $p \leq 0.001$; *** $p \leq 0.0001$.

4.4 Conclusions

Considering all the assays, CTAB proved to have the global strongest biocidal effect on both filamentous strains. Even if in the XTT reduction assay triclosan had a higher toxic effect to *S. natans,* the overall biocidal effect, considering all results, was that of CTAB. Anyway, it must be noted that triclosan had stronger effects in the defilamention than CTAB and in the case of *N. amarae,* CTAB did not alter the filamentation in the first 24 h.

Differences in susceptibility to the exposure of the tested biocides suggested strain-dependent effects. Comparing both strains, *N. amarae* showed to be less susceptible to triclosan and glutaraldehyde than *S. natans*, although *N. amarae* is Gram positive and *S. natans* is Gram negative, contradicting the literature. It was observed that the range of biocide concentrations for *N. amarae* was about 5-fold to 25-fold higher for glutaraldehyde and triclosan, respectively. CTAB had a similar effect on both strains on what dose range is respected, but the epifluorescence microscopy revealed that CTAB did not affect filamentation of *N. amarae* significantly in the first 24 h of exposure.

The results of the different assays were rather complementary than contradictory and allowed to corroborate the different mechanisms that lie in the action of the three biocides. It is also clear that the results on viability, possibility of recovery and fragmentation of filaments must be taken in account considering the species and the foreseen effects in the cases of filamentous overgrowth.

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4.6 Supplementary materials

The mean percentage values, SD and SEM of controls and biocides concentrations after 24 h, 72 h of biocide treatment and after recovery assay at 72 h are shown in Table 4.13 for *N. amarae* (live, injured and dead cells) and in Table 4.14 for *S. natans* (live, injured and dead cells).

Table 4.3: Mean percentage values (%), SD and SEM of controls and biocides concentrations after 24 h, 72 h of biocide treatment and after recovery assay at 72 h for live, injured and dead *N. amarae* cells

24 h	Live			Injured			Dead		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
control	**94.30**	4.00	1.40	**4.03**	3.30	1.20	**1.64**	0.80	0.30
DMSO	**85.00**	19.40	7.30	**11.97**	16.90	6.40	**3.03**	2.70	1.00
TL	**82.10**	9.50	3.00	**9.78**	4.30	1.40	**8.08**	4.50	1.40
TH	**13.44**	10.80	2.70	**62.56**	27.50	6.90	**24.00**	23.90	6.00
CL	**62.47**	12.80	9.00	**14.83**	5.70	4.00	**22.70**	7.10	5.00
CH	**1.64**	1.70	0.60	**47.38**	43.20	15.30	**50.98**	43.80	15.50
GL	**74.83**	14.60	8.40	**18.20**	13.00	7.50	**6.97**	2.30	1.30
GH	**75.95**	19.80	7.50	**20.06**	16.30	6.20	**3.99**	3.70	1.40
72 h	Live			Injured			Dead		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
control	**52.53**	23.70	9.00	**25.00**	14.20	5.40	**22.40**	20.50	7.80
DMSO	**53.70**	5.20	2.00	**19.10**	7.90	3.00	**27.20**	26.60	10.10
TH	**25.32**	20.30	5.60	**57.50**	19.50	5.40	**17.20**	13.90	3.80
TR	**24.73**	18.70	5.00	**52.60**	15.40	4.10	**22.70**	25.60	6.80
CH	**7.47**	9.80	4.90	**79.90**	15.10	7.50	**12.80**	17.60	8.80
CR	**17.93**	14.20	7.10	**76.30**	39.90	14.10	**5.80**	5.10	2.60
GH	**16.64**	4.50	2.30	**76.40**	1.50	0.70	**7.00**	4.90	2.50
GR	**36.35**	12.10	6.10	**59.90**	10.00	5.00	**3.80**	2.20	1.10

Table 4.4: Mean percentage values (%), SD and SEM of controls and biocides concentrations after 24 h, 72 h of biocide treatment and after recovery assay at 72 h for live, injured and dead *S. natans* cells

24 h	Live			Injured			Dead		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
control	**86.40**	8.10	3.30	**6.98**	2.30	0.90	**6.62**	7.30	3.00
DMSO	**84.24**	10.70	4.30	**9.27**	2.90	1.20	**6.49**	8.40	3.40
TL	**77.83**	12.50	3.90	**9.18**	2.00	0.60	**12.99**	11.90	3.70
TH	**61.95**	24.80	7.90	**12.61**	4.20	1.30	**25.44**	22.20	7.00
CL	**88.96**	4.80	1.40	**6.47**	1.50	0.40	**4.57**	4.90	1.40
CH	**2.89**	1.70	0.50	**36.12**	11.60	3.30	**60.99**	11.20	3.20
GL	**74.71**	8.20	2.90	**10.93**	3.50	1.20	**14.36**	10.10	3.60
GH	**56.83**	26.60	8.90	**15.14**	5.10	1.70	**28.03**	22.10	7.40
72 h	Live			Injured			Dead		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
control	**75.80**	13.83	5.23	**14.25**	11.21	4.24	**9.95**	7.57	2.86
DMSO	**77.83**	14.51	5.92	**14.50**	13.97	5.71	**7.67**	3.12	1.27
TH	**13.26**	21.80	6.29	**10.11**	4.93	1.42	**76.63**	25.41	7.34
TR	**26.57**	24.01	6.93	**10.00**	5.16	1.49	**63.43**	25.65	7.41
CH	**0.99**	2.26	0.72	**5.02**	3.98	1.26	**93.99**	5.87	1.86
CR	**14.06**	15.66	5.22	**8.79**	4.36	1.45	**77.15**	17.82	5.94
GH	**41.85**	25.59	8.09	**27.18**	11.66	3.69	**30.97**	18.10	5.72
GR	**56.43**	19.79	6.26	**19.07**	5.92	1.87	**24.50**	17.98	5.69

Box and whiskers plots were displayed to compare the effect of the highest concentration of biocides on live bacteria *vs* time of treatment. In Figure 4.10 and Figure 4.11 are present the data for *N. amarae* and *S. natans*, respectively.

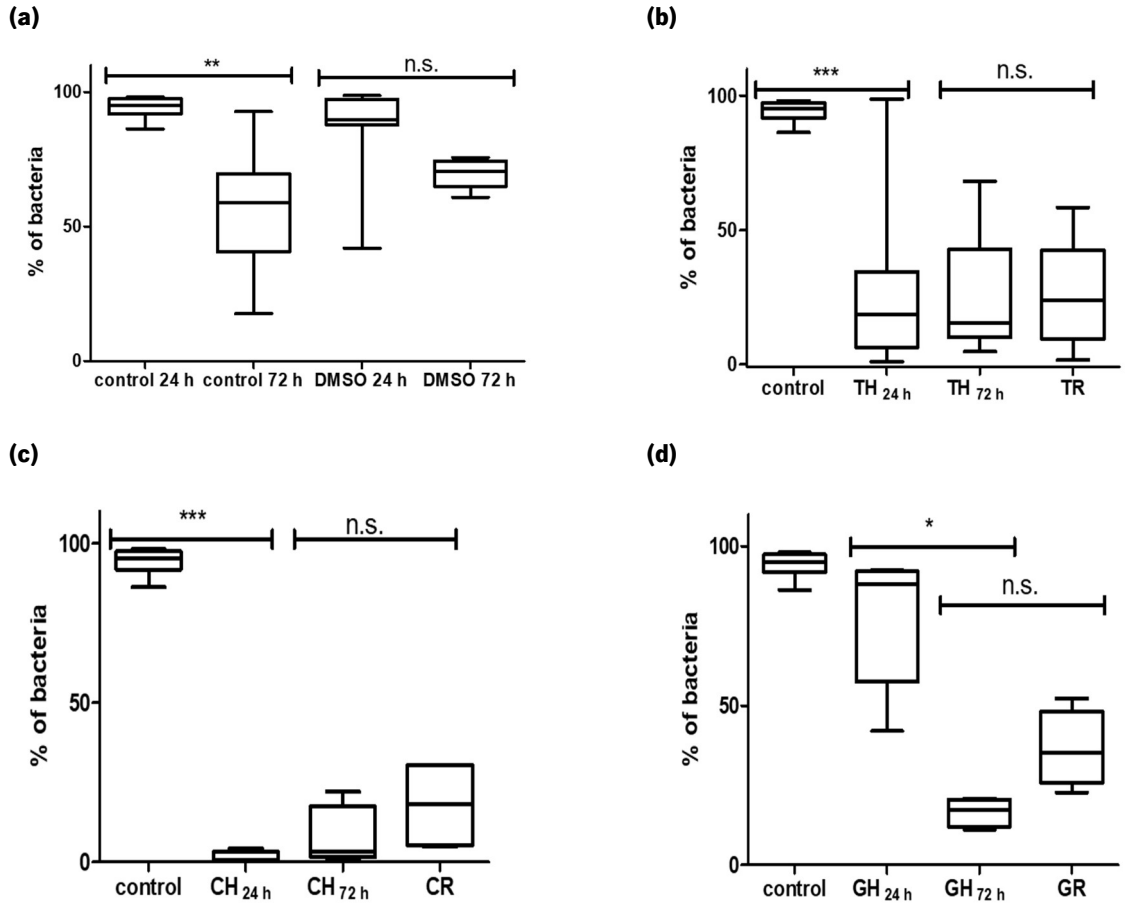

Figure 4.10: Comparison of live *N. amarae* cells (%) present in the **(a)** controls (0 µg/mL and DMSO) and in the highest concentration (µg/mL) of **(b)** triclosan (TH $_{24\,h}$, TH $_{72\,h}$, TR), **(c)** CTAB (CH $_{24\,h}$, CH $_{72\,h}$, CR) and **(d)** glutaraldehyde (GH $_{24\,h}$, GH $_{72\,h}$, GR) respectively, after 24 h, 72 h of biocide treatment and after recovery assay at 72 h. All the columns show the mean ± SEM (n=3). n.s. Not statistically significant difference with the DMSO control of 24 h and 72 h, with TH $_{72\,h}$ and TR, with CH $_{72\,h}$ and CR and with GH $_{72\,h}$ and GR; Statistically significant difference with GH $_{24\,h}$ and GH $_{72\,h}$: * $p \leq 0.05$; Statistically significant difference with control (0 µg/mL) of 24 h and 72 h: ** $p \leq 0.001$; Statistically significant difference with control (0 µg/mL) and TH $_{24\,h}$ and with control (0 µg/mL) and CH $_{24\,h}$: *** $p \leq 0.0001$.

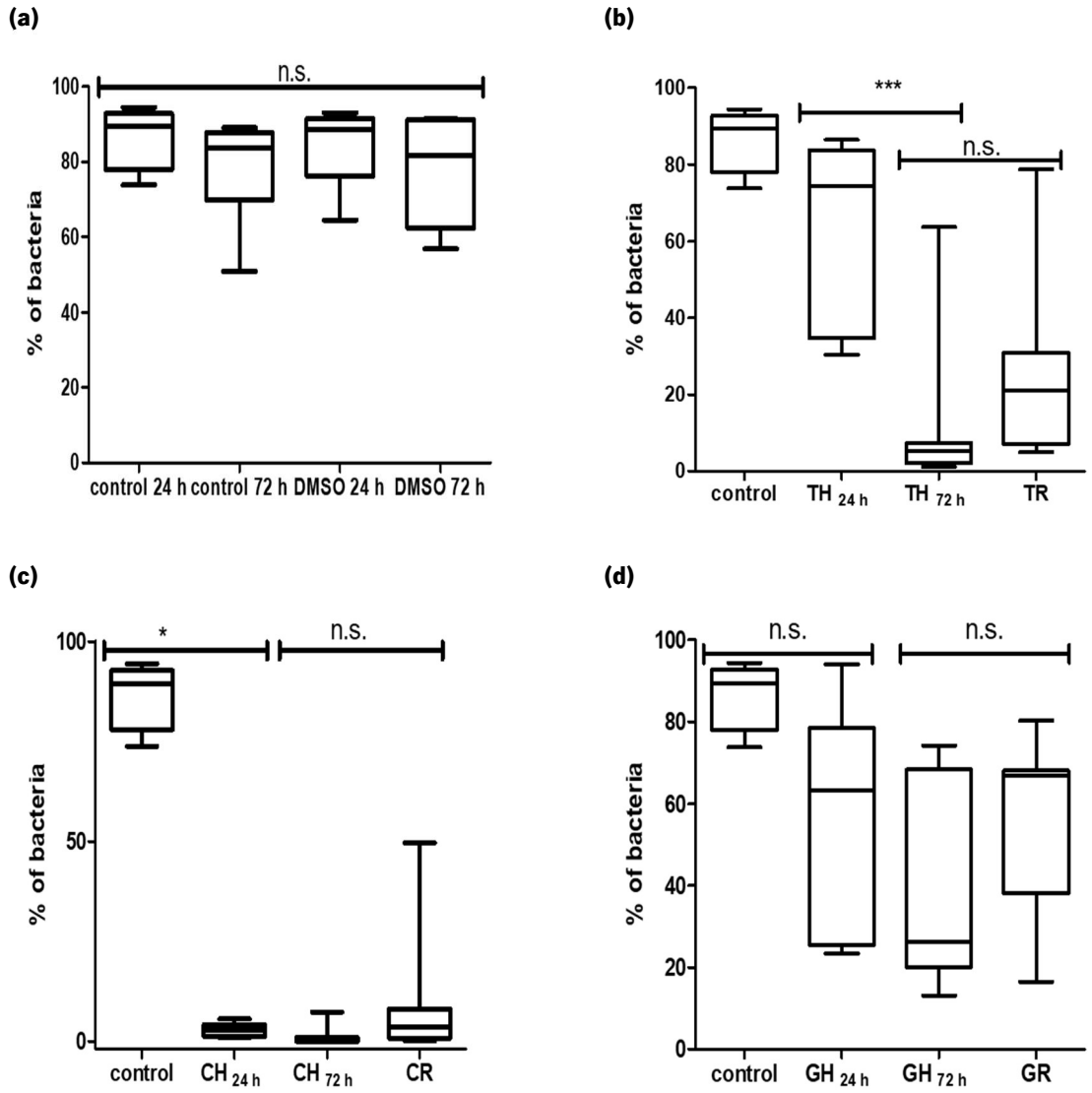

Figure 4.11: Comparison of live *S. natans* cells (%) present in the **(a)** controls (0 µg/mL and DMSO) and in the highest concentration (µg/mL) of **(b)** triclosan (TH $_{24\,h}$, TH $_{72\,h}$, TR), **(c)** CTAB (CH $_{24\,h}$, CH $_{72\,h}$, CR) and **(d)** glutaraldehyde (GH $_{24\,h}$, GH $_{72\,h}$, GR) respectively, after 24 h, 72 h of biocide treatment and after recovery assay at 72 h. All the columns show the mean ± SEM (n=3). n.s. Not statistically significant difference with all the controls of 24 h and 72 h, with TH $_{72\,h}$ and TR, with CH $_{72\,h}$ and CR and with control and GH $_{24\,h}$, GH $_{72\,h}$, and GR; Statistically significant difference with the control (0 µg/mL) and CH $_{24\,h}$: * $p \leq 0.05$; Statistically significant difference between TH $_{24\,h}$ and TH $_{72\,h}$: *** $p \leq 0.0001$.

CHAPTER 5

Evaluation of biocides action on the microbial community and on the performance of activated-sludge

While in the previous chapter the effect of various biocides on two filamentous bacteria was studied *in-vitro*, this chapter will evaluate the effect of the same biocides – triclosan, CTAB and glutaraldehyde - in conditions close to reality, using activated-sludge collected from WWTP, bearing in mind that the latter situation is far more complex than the previous one, due to the multiplicity of strains and environmental factors co-existing in a real activated-sludge.

Mixed liquor and real sewage were collected from three WWTP, the biocides effect being initially assessed in respirometric assays. Furthermore, the biocide effect was also assessed in bench-scale trials, but in this case with mixed liquor and real sewage from only one of the WWTP. In the respirometric assays, all the biocides showed a dose-dependent effect on biomass respiration inhibition: triclosan caused the strongest inhibition, followed by glutaraldehyde and CTAB, by this order if considering the initial mass concentrations but in the reversed order (between glutaraldehyde and CTAB) if molar concentrations are to be considered. In the overall performance bench-scale assays (with only Braga WWTP mixed liquor), all the tested biocides led to toxic effects in the microfauna, at the used concentrations along the 72 hours of treatment. A biocidal effect was perceptible for triclosan at the lowest concentrations, and for CTAB and glutaraldehyde the effect was only significant at higher concentrations. Focusing in the control of the excess of filamentous bacteria, the Gram negative Type 0092 was more susceptible to triclosan than to CTAB and glutaraldehyde in the tested range of concentrations. For the reduction of Gram positive (Type 0041/0675), all three biocides reduced it one value in the score, but both CTAB and glutaraldehyde were effective independent of concentration and time of incubation. In the live/dead viability assays, it was shown that biocides addition gave rise to fragmentation of filamentous bacteria into single cells and loss of floc structure (lower density). Triclosan and CTAB showed a clear fragmentation effect, mainly on the surface of the flocs, since the beginning and this effect remained till the end of the assay.

This chapter is based on the following submitted publication: Ferreira, V., Dias, N., Mota, M., Pereira, M.O., Nicolau, A., 2022. Evaluation of biocides action on microbial community and on the performance of activated-sludge. (Submitted to *Applied and Environmental Microbiology*).

5.1 Introduction

The activated-sludge wastewater treatment is based on the activity of a bacterial aerobic community suspended in the aeration tank, and fed with fresh effluents containing biodegradable organic substances required for its development. The presence of the bacterial community thus also allows the growth of a microfauna consisting mainly of grazing organisms such as protozoa and small metazoan (Amaral *et al.* 2004). Among protozoa, ciliates acquire special significance as they consume most of the dispersed bacteria in the mixed-liquor and consequently take part in the reduction of suspended solids and turbidity in the final effluent (Al-Shahwani & Horan 1991; Martín-Cereceda *et al.* 1996). Moreover, they are especially sensitive to changes in environmental conditions and can be used as biological indicators and it has been used all over the years in different ways, allowing for the evaluation of either the conditions of the aeration tank or even the performance of the system (Hailei *et al.* 2017). Methods based on protozoan population structure have been used to assess activated-sludge plant performance, such as the Sludge Biotic Index (SBI) proposed by Madoni (1994). SBI is based on the abundance and diversity of the community of protozoa and small metazoan and on the different sensitivities revealed by several microfauna groups, with physical-chemical and operational parameters prevailing in activated-sludge system.

The performance of the activated-sludge process largely depends on the balance between filamentous and floc-forming bacteria (Seviour & Blackall 1999; Tandoi *et al.* 2017). The abundance of filamentous bacteria in activated-sludge can be measured according to the subjective scoring method of Jenkins *et al.* (2004) where the observations are rated on a scale from 0 (none) to 6 (excessive). When the normal balance of the community is disturbed, filamentous bacteria tend to proliferate causing various problems, namely bulking and foaming phenomena, referenced as the major problems of the activated-sludge systems, resulting in poor sedimentation of sludge and low quality final effluents (da Motta *et al.* 2003; Tsang *et al.* 2008).

Control of filament proliferation enhances sludge settleability and can be promoted by specific and/or non-specific methods. Specific methods are preferable to control filamentous bulking, because the involve a proper identification and characterization of the specific filamentous bacteria within a bulking sludge, thereby determining the causes of its occurrence (Zhang *et al.* 2017; Liu *et al.* 2019). Non-specific methods are only temporary solutions and, as they do not remove the causes of the excessive growth, they might be potentially detrimental to the biomass as a whole (both filamentous and floc-forming microorganisms) (Caravelli *et al.* 2003; Henze *et al.* 2008; Liu *et al.* 2019).

An example of the non-specific methods is the addition of chemical substances such as biocides that allow to inhibit or inactivate the growth of organisms such as algae, bacteria and fungi (Singh 2017). The addition of biocides is, because of the fastness of their action and practicability, one of the most commonly used methods to control filamentous bulking under acute conditions (Jenkins *et al.* 2004). Biocides exert lethal or inhibiting effects by interacting with one or more target cell sites. The target sites include the peptidoglycan layer, cytoplasmic membrane, outer membrane, structural proteins, thiol groups of enzymes and bacterial spore coats (Bitton 2005; Davies 2015; Singh 2017). The correct choice of addition points and appropriate amounts of addition should be carefully determined (Guo *et al.* 2012). In this study, the three biocides (triclosan, CTAB and glutaraldehyde) tested in Chapter 4 were chosen to evaluate the biocidal effect both in the microbial community and in the performance of activated-sludge.

Toxicant effects can be quite complex and not easily predictable and may lead to collateral effects such as the inhibition of waste organic biodegradation and to the reduction of solid-liquid separation efficiency in the aerated tank (Ricco *et al.* 2004; Çeçen & Tezel 2017). The aerobic degradation of organic material can be determined by measuring the oxygen uptake rate (OUR) by microorganisms, usually referred to as respirometry. OUR measurements can deliver information concerning treatment plant performance or wastewater characteristics, to monitor, optimize and troubleshoot the activated-sludge processes. So, respirometry can be used as a toxicity test for detection of inhibitory streams (Bonté *et al.* 2005; Jurga *et al.* 2017). The first authors that focused in the respirometric technique to this use were Jenkins (1959) and Montgomery (1967) based on their own experimental studies on the quantification of DO consumption in activated-sludge systems. The procedure for estimating the rate of oxygen consumption is extremely simple and the field of application vast. The OUR test considers variations in the respiration rate of the mixed-liquor as a consequence of the type of substrate added and the rate of degradation by the biomass. The rate of oxygen consumption can be determined in the presence or absence of external carbon and energy sources to the microorganisms. Whenever there are no sources present, oxygen consumption is due to endogenous metabolism, leading to measure the endogenous rate of respiration. In the presence of carbon and energy sources, the oxygen consumption is due to oxidation reactions, referred to as the total respiration rate. A discontinuous operation respirator allows the evolution of oxygen concentration to be recorded over time. This record is called a respirogram (Pereira 2001). In the present study, the method was carried on by withdrawing a sample of activated-sludge from a WWTP, transferring it into a small vessel, aerating it, ceasing the aeration and then monitoring the decline of DO concentration with time (Jurga *et al.* 2017). The relationship between the decrease in oxygen concentration and time is

normally found to be linear as in Figure 5.1 and OUR is derived from the slope of DO decline (Hao *et al.* 2010).

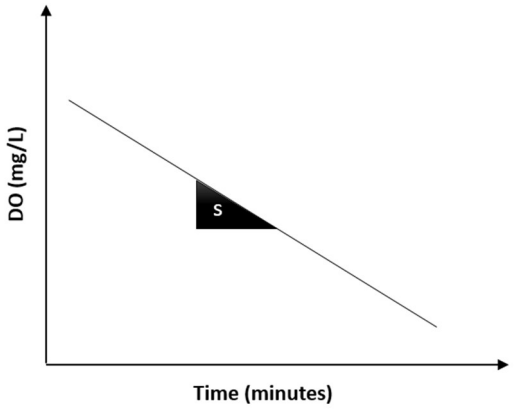

Figure 5.1: Graphical representation of the DO profile; S = slope

OUR is usually converted to specific oxygen uptake rate (SOUR) to give a more accurate representation based on the concentration of microorganisms in sludge culture. SOUR can be calculated by simply dividing OUR by mixed liquor volatile suspended solids (MLVSS). As SOUR measures aerobic activity it has an interesting informative capacity – it can be used as a toxicity test – because this measurement permits following the instantaneous sludge answer to injection of toxics (inhibition test) (Madoni *et al.* 1999; Basnyat 2011).

Studies on the fate of biocides along wastewater treatment processes are scarce (Petrie *et al.* 2015). Nevertheless, they are seen as promising in scientific research, since they may help to know how much sub-lethal concentrations of biocides can affect the microbial community and the overall performance of activated-sludge systems, and whether their presence can be used to filamentous bulking control.

In the present study, respirometric assays were carried on with the mixed liquor and real sewage of three WWTP, followed by a bench-scale assay with mixed liquor and sewage from only one of the WWTP, after the exposure to three biocides – triclosan, CTAB and glutaraldehyde.

Similar to the previous chapter, the present study was initially conceived with mg/g MLSS metrics, but conclusions are withdrawn based also on µmol/g MLSS.

5.2 Experimental procedures

5.2.1 Experiments performed

Respirometry assays were conducted during this study, using real mixed liquor and real sewage from three different WWTP, all of them located in Braga, Portugal, from the Municipal Wastewater Treatment Plant of Braga, the industrial (compact) Celeirós WWTP and the domestic (compact) Arentim WWTP, respectively. Bench-scale overall community assays were conducted in Erlenmeyer 500 mL flask using mixed liquor and real sewage only from Braga WWTP.

Mixed-liquors (activated-sludge) were collected directly from the aeration tank as *innocula* and real sewage filtrates collected after primary treatment (i.e., after the removal of solids, sands and grease and primary sedimentation) to feed the *innocula*. To prevent anoxia, the activated-sludge was aerated during transportation (within 30 minutes) to the laboratory where they have been constantly aerated until the end of the assay with aeration pumps (aquarium air pumps). All activated-sludge samples collected were kept at the temperature of the aeration tank from which it was drawn until the end of study.

Operation parameters in the experimental assays for the three WWTP studied are present in Table 5.1.

Table 5.1: WWTP operational parameters

	Braga WWTP	Celeirós WWTP	Arentim WWTP
pH*	7.5	6	7
DO * (mg/L)	2.5	1.5	2
Temperature (°C)	20	20	20
MLSS* (mg/L)	4765	3992	3260
MLVSS * (mg/L)	3885	2989	2643

* mean value

5.2.2 Biocides

Triclosan (97 % pure) was purchased from Aldrich Chemicals (USA). The triclosan stock solution, was prepared using DMSO, as a solvent. As DMSO affects cell viability at concentrations above 1.5 % (Chen & Thibeault 2013; Yuan *et al.* 2014), a preliminary test (data not shown) was carried out to verify the maximum possible volume of DMSO (10 mg/mL). CTAB was purchased from Acros Organics (USA) (99 % pure) and the stock solution was prepared in distilled water. Glutaraldehyde solution 25 % in H_2O (density 1.062 g/mL) was purchased from Aldrich Chemicals and the stock solution was also prepared

in distilled water. All biocide stock solutions were prepared on WWTP weight basis (MLVSS): 28 g Biocide/L for Braga WWTP and 21 g Biocide/L for Celeirós WWTP and Arentim WWTP, respectively.

One control (without biocide) and the six different concentrations of biocides (as shown in Table 5.2) were tested at two end-points (30 minutes and 3 h) for the respirometry assay and four end-points (0 h, 24 h, 48 h and 72 h) for the bench-scale assays. Two independent replicates assays were performed for each biocide concentration and an average value was calculated. The biocides were added only at the beginning of the test (time = 0 h). Targeted concentrations were obtained by adding a variable volume of triclosan, CTAB and glutaraldehyde stock solution, respectively.

Table 5.2: Concentrations of biocides

	Concentration (mg/g MLSS)						
Triclosan							
CTAB	0	2	5	10	20	30	50
Glutaraldehyde							

5.2.3 Experimental assays

5.2.3.1 Respirometry assay

The impact of each biocide on sludge activity (toxicity test) was assessed by respirometry, which was based on the experimental protocol described on EPA METHOD 1683 (2001), by Mrafková *et al.* (2003) and Santos (2012). The protocol was adapted for the experimental conditions. OUR and SOUR were measured at each biocide concentration in a self-made respirometer (batch respiration meter) with an internal volume of 300 mL. Sewage, mixed liquor and biocides (test-material) were all added to the bottles in the proportions shown in Table 5.3. Activated-sludge was used only for one test at a time, i.e., the three biocides tests (from 0 mg/g MLSS to 50 mg/g MLSS concentration) were always performed with fresh samples of renewed activated-sludge to avoid acclimatization of the biomass to the biocide, thereby avoiding underestimation of the biocide effect. Consequently, during the whole experimental period, different samples (from the three WWTP) were used (avoiding the use of samples stored for long periods of time).

Table 5.3: Respirometric test set-up

	Braga WWTP	Celeirós WWTP	Arentim WWTP
Activated-sludge (mL)	280	280	280
Sewage filtrate (mL)	15	15	15
Biocide solution (mL)	5	5	5
Distilled water (mL) *	5	5	5
Final volume (mL)	300	300	300

* only for controls

During the experiments, it was only possible to make one OUR measurement at a time; to test the different initial concentrations of biocides, the control test was first carried out, followed by the different concentrations to be tested. Each experiment was performed on an Erlenmeyer 500 mL flask used as activated-sludge unit, receiving 280 mL of mixed-liquor, and continuously aerated for at least 30 minutes to reach a state of endogenous respiration and to degrade any substrate absorbed by the activated-sludge.

The temperature (20 °C) and rotation of the magnetic stirrer (300 rpm) were controlled along the assay. At the beginning of the experiment, microscopic examination of the microbial community from the activated-sludge was performed and concentration of MLVSS and pH were measured. When necessary, pH was adjusted to a range between 7 and 7.5 by addition of hydrochloric acid (HCl) and/or sodium hydroxide (NaOH) solutions. Therefore, sewage filtrate and biocide (at different concentrations) were added to the 500 mL activated-sludge unit (in the proportions presented in Table 5.3) and aerated for 10 minutes. The same procedure was adopted for the control, but no biocide was added. After the contact time, the aeration was stopped and the volume of the activated-unit was placed in the respirometer (volume = 300 mL). The DO was then measured for 15 minutes or until DO < 1.5 mg/L, with the DO electrode hermetically sealed to the test respirometer bottle (using a rubber stopper attached to the probe), to determine the OUR. The DO electrode was connected to a dissolved oxygen meter (Hanna, HI 764080 model, USA) which allowed the acquisition of the data over time. The DO probe was calibrated prior to each respirometric experiment. After the first end-point, the activate-sludge analysed above was transferred back into the Erlenmeyer 500 mL flask used as activated-sludge unit and placed under continuous aeration up to 3 h. Afterward, a volume of sewage filtrate (Table 5.3) was added again to the activated-sludge unit and aerated with a 10-minute contact time. The aeration was then stopped and the volume of the activated-unit was placed in the respirometer. The subsequent procedure was the same as the one previously described: DO was measured during 15 minutes or until DO < 1.5 mg/L, with the DO electrode hermetically sealed to the test respirometer bottle (using a rubber stopper attached to the

probe), to determine OUR. The comparison of the control and the biocide OUR measurements, during the following 15 minutes for the two end-points (30 minutes and 3 h), gives the instantaneous inhibition effect of the toxic on the biomass. The laboratory set-up used in this assay is shown in Figure 5.2.

(a) **(b)**

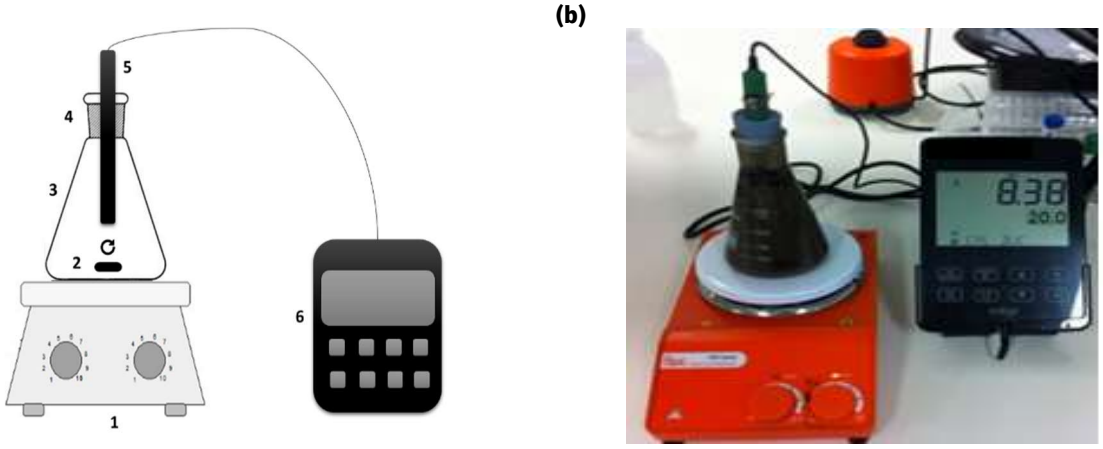

Figure 5.2: (a) Schematic picture of the laboratory set-up for OUR measurements: (1) Hot plate stirrer; (2) Magnetic stirrer; (3) Test respirometer; (4) Sealing stopper; (5) DO electrode; (6) DO meter and data acquisition. **(b)** System used for the respirometric assay.

OUR could be calculated as the slope of DO plot *vs* time by linear regression, based on following equation (Eq. 5.1):

$$\boldsymbol{OUR = \frac{DO_1 - DO_2}{\Delta t}} \qquad \text{Eq. 5.1}$$

where OUR is the oxygen uptake rate (mg/L.h); DO_1 and DO_2 is the dissolved oxygen concentration (mg/L) at the start and finish of the test, respectively; Δt is the test duration time expressed in hours.

SOUR, as specific respiration rate, was calculated using equation (Eq. 5.2), where X is the MLVSS (mg/L). The inhibitory effect of the tested biocide at each concentration and the control, expressed as percent inhibition, was calculated as (Eq. 5.3):

$$\boldsymbol{SOUR = \frac{DO_1 - DO_2}{X\,\Delta t} = \frac{OUR}{X}} \qquad \text{Eq. 5.2}$$

$$\boldsymbol{\%\ Inhibition = \left(1 - \frac{SOUR_t}{SOUR_c}\right) \times 100} \qquad \text{Eq. 5.3}$$

where $SOUR$ is the specific oxygen uptake rate (mg/g MLVSS.h); X is the MLVSS concentration (mg/L). $SOUR_c$ and $SOUR_t$ are the specific respiration rate of the control and of the tested biocide concentration, measured after 30 minutes (min) and 3 hours (h), respectively.

Half maximal inhibitory concentration (IC_{50}) is a measure of the capacity of a substance to inhibiting a specific biological or biochemical process. It is the most widely used and informative measure of a substance (inhibitor) efficacy and is usually experimentally determined by exposing a tested population to an inhibitor (Aykul & Martinez-Hackert 2016). IC_{50} defines the biocide concentration required to inhibit a given biological process of the tested population by half (50 %), after 30 min and 3 h of exposure. Linear regression analysis was used to determine IC_{50} values for triclosan, CTAB and glutaraldehyde.

5.2.3.2 Bench-scale assay

The evaluation of biocides action on the microbial community and on the performance of activated-sludge treatment process was assessed at bench-scale, and was based on the experimental protocol described by Yuan *et al.* (2015). The protocol was adapted for the current experimental conditions.

The evaluation was made for each biocide concentration in a 500 mL Erlenmeyer flask. A preliminary test was carried out with several volumes of mixed liquor and sewage (in a total volume of 300 mL) during 72 h, in order to choose the volume that remained more stable in terms of protozoa and filamentous bacteria. After this preliminary assay, sewage, mixed liquor and biocides were all added to the Erlenmeyers in the proportions shown in Table 5.4. As in the respirometry experiments, in the bench-scale assays, each mixed liquor sample was used only for one single test, *i.e.,* the tests with all the biocides (from 0 mg/g MLSS to 50 mg/g MLSS concentration) were always performed with fresh samples of mixed liquor to avoid acclimatization of the biomass to the biocide, in this way avoiding the underestimation of the biocidal effect. Also, during the whole experimental period, different municipal mixed liquor samples from Braga WWTP were used, to avoid the use of sludge stored for very long periods of time.

Table 5.4: Bench-scale test set-up

	Volume
Activated-sludge (mL)	255
Sewage filtrate (mL)	40
Biocide solution (mL)	5
Distilled water (mL) *	5
Final volume (mL)	300

* only for controls

Seven parallel 500 mL Erlenmeyer flasks were used as activated-sludge units, one control and six with the defined concentration of each of the biocides, each having a working volume of 300 mL continuously aerated. During the experiment, a cotton stopper was placed on each Erlenmeyer flask to minimize evaporation. The temperature (20 °C) and rotation of the magnetic stirrer (80 rpm) were controlled along the assay. At the beginning of the experiment, the activated-sludge pH from Braga WWTP was measured and adjusted to a range between 7 and 7.5 by addition of HCl and NaOH solutions when necessary. Afterward, a mix of mixed- liquor, sewage filtrate and biocide (in the proportions present in Table 5.3) were placed in all seven Erlenmeyer flasks, with the system stirred and aerated. Activated-sludge units were sampled at four end-points (0 h, 24 h, 48 h and 72 h) for physical-chemical, operational analyses, and biocidal assessment. It should be noted that the sample identified as 0 h was actually collected after 30 minutes of contact (maximum) due to logistic constraints. The laboratory set-up used is shown in Figure 5.3.

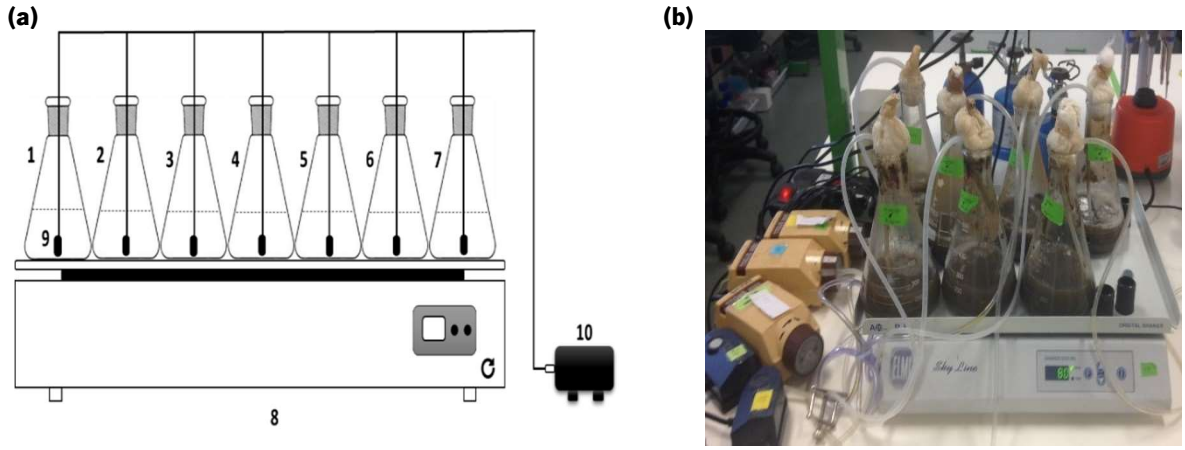

Figure 5.3: (a) Schematic picture of the laboratory set-up for bench-scale assays: (1) Control test; (2-7) Biocide concentration tests; (8) Orbital shaker; (9) Air stone oxygen diffuser; (10) Aquarium air pump (x 7). **(b)** System used for the bench-scale assay.

5.2.4 Microbiological analysis

Protozoa and small metazoan were identified (at the genus or species level) by using an optical microscope CX41 (Olympus, Japan) (100 X to 400 X magnifications) and with the help of identification guides (Madoni 1994; Madoni *et al.* 2005). A sample volume of 25 µL from each activated-sludge unit was collected with an automatic piston-micropipette in duplicate to be observed and analysed. The SBI calculation was achieved according to the guidelines of Madoni (1994) and values range from 0 (indicating the poorest condition) to 10 (indicating the best condition). The SBI values are grouped into four classes corresponding to different levels of biological quality of the sludge. Class I (values 8 to 10) corresponds

to highly colonized and stable sludge, excellent biological activity, and good performance. Class II (values 6 and 7) corresponds to well colonized and stable sludge, with biological activity in decline and good performance. Class III (values 4 and 5) corresponds to insufficient biological depuration in the aeration tank and mediocre performance. Finally, class IV (values 0 to 3) corresponds to poor biological depuration in the aeration tank and low performance of the plant.

Fresh samples from each activated-sludge unit, were prepared for examination of the filamentous bacteria. To determine the morphological characteristics of the various filaments, samples were inspected using an optical microscope CX41 (Olympus, Japan) under bright-field and phase contrast (1000 X magnification) together with identification guides when necessary (Eikelboom 2000; Jenkins *et al.* 2004). The smears of each collected fresh sample were stained according to the staining techniques of Gram and Neisser and examined under immersion in oil and bright-field (1000 X magnification). Multiple images were captured with an SC30 digital camera (Olympus, Japan). The abundance of filamentous microorganisms was quantified according to a subjective scoring of filament abundance (0 = none, 1 = few, 2 = some, 3 =common, 4 = very common, 5 = abundant and 6 = excessive) adapted from (Jenkins *et al.* 2004).

5.2.5 Physical-chemical and operational parameters

The activated-sludge units were sampled every day (0, 24, 48 and 72 h) for physical-chemical and operational analysis according to the *Standard Methods* (APHA 1998). Regular analysis included determinations of pH, dissolved oxygen (DO), mixed-liquor suspended solids (MLSS) and mixed-liquor volatile suspended solids (MLVSS).

5.2.6 Live/dead viability test (Epifluorescence microscopy)

Dyes and staining conditions

SYTO-BC and PI were purchased from Molecular Probes, Invitrogen, Eugene, OR, USA. SYTO-BC stock solution was prepared at 5 μM in pre-filtered (0.2 μm) sterilized water and PI was used at 1.0 mg/mL. Stock solutions were stored frozen at –20 °C and protected from light.

Bacterial preparation and staining conditions

A volume of 2 mL bacterial cells (sample) of each concentration studied was concentrated by centrifugation at 10,000 × g for 1 minute. The supernatant was removed and the pellet was re-suspended in 2 mL of pre-filtered 0.85 % NaCl buffer. The samples were pelleted again by centrifugation at 10,000 × g for 1 minute. The supernatant was removed and the pellet was re-suspended in 500 µL of pre-filtered 0.85% NaCl buffer.

Staining conditions for live/dead viability assay were as following: dyes were added to the bacterial suspension at a final concentration of 0.30 µM of SYTO-BC and 1.5 µM of PI.

Epifluorescence microscopy procedure

The samples were incubated in the dark for 10 minutes and analysed with an epifluorescence microscope BX51 (Olympus, Japan) using FITC (EX 470 – EM BP490/520) and TRITC (EX 530 – EM BP550/590) filters to visualise live and dead cells respectively. All images were taken using *Citifluor* non-fluorescent immersion oil (Agar scientific, UK) containing antifadent AF87, designed for fluorescent microscopy (high magnifications) to reduce the fading of fluorescence dyes used for labelling biological species. Multiple images were captured with a DP72 digital camera (Olympus, Japan) and acquired using Olympus CellSens standard software 1.16 version.

SYTO-BC and PI double-stained control was also observed under epifluorescence microscopy.

5.2.7 Statistical analysis

Statistical data analysis, comparisons and graphics were performed in Microsoft Excel (USA) and GraphPad Prism software 5.0 version (USA). The two-way analysis of variance (ANOVA) was performed using GraphPad Prism. Cell inhibition was expressed as mean ± standard error (SEM, n = 2). The normal distribution of data for the parametric test ANOVA was confirmed using the Kolmogorov-Smirnov test, D'Agostino-Pearson omnibus normality test and Shapiro-Wilk normality test. The non-normally distributed data were analysed using the non-parametric test Kruskal-Wallis. Post-hoc tests were used to confirm significant differences between groups. Bonferroni multiple comparison test and Dunn´s multiple comparison tests were carried out for the parametric and non-parametric tests, respectively. Statistically significant differences were determined for p-values ≤ 0.05(*), 0.01(**), and 0.001(***).

5.3 Results and discussion

5.3.1 Respirometry assay

The biocide effect was proportional to the reduction of OUR and SOUR indicating an increase of bacterial inhibition, as expected. The specific oxygen uptake rate (SOUR) of the sludge microorganisms and the inhibition percentage in Braga, Celeirós and Arentim WWTP are presented in Table 5.5, Table 5.6 and Table 5.7, respectively.

Table 5.5: Biocide concentrations, Specific oxygen uptake rate (SOUR), Standard deviation (SD) and Inhibition percentage (mean values) measured after both 30 minutes and 3 h of biocide exposure in the Braga WWTP activated-sludge samples (two independent replicates assays)

Biocide	Concentration (mg/g MLSS)	After 30 min			After 3 h		
		SOUR (mg/g MLVSS.h)	SD	Inhibition (%)	SOUR (mg/g MLVSS.h)	SD	Inhibition (%)
Triclosan	0 *	16.50	0.71	0	15.50	0.71	0
	2	18.50	0.71	-12	14.00	0.00	10
	5	13.50	0.71	18	9.50	0.71	39
	10	9.50	2.12	42	6.50	0.71	58
	20	6.00	0.00	64	4.50	0.71	71
	30	4.50	0.71	73	3.50	0.71	77
	50	3.00	0.00	82	2.00	1.41	87
CTAB	0 *	14.00	4.24	0	13.00	4.24	0
	2	16.00	4.24	-14	11.50	2.12	12
	5	13.00	1.41	7	9.00	1.41	31
	10	11.00	1.41	21	8.00	1.41	38
	20	9.50	0.71	32	6.50	0.71	50
	30	8.00	1.41	43	5.50	0.71	58
	50	5.50	0.71	61	3.50	2.12	73
Glutaraldehyde	0 *	14.00	4.24	0	11.5	3.54	0
	2	11.50	4.95	18	9.00	2.83	22
	5	11.00	4.24	21	9.00	2.83	22
	10	10.00	4.24	29	7.50	3.54	35
	20	9.00	2.83	36	5.50	2.12	52
	30	7.00	2.83	50	3.50	0.71	70
	50	5.00	1.41	64	2.50	0.71	78

* control sample

Table 5.6: Biocide concentrations, Specific oxygen uptake rate (SOUR), Standard deviation (SD) and Inhibition percentage (mean values) measured after both 30 minutes and 3 h of biocide exposure in the Celeirós WWTP activated-sludge samples (two independent replicates assays)

Biocide	Concentration (mg/g MLSS)	After 30 min			After 3 h		
		SOUR (mg/g MLVSS.h)	SD	Inhibition (%)	SOUR (mg/g MLVSS.h)	SD	Inhibition (%)
Triclosan	0 *	16.00	0.00	0	13.00	0.00	0
	2	17.5	0.71	-9	10.00	1.41	23
	5	9.50	2.12	41	6.50	2.12	50
	10	6.50	2.12	59	5.00	1.41	62
	20	5.50	2.12	66	4.00	1.41	69
	30	3.50	0.71	78	2.00	0.00	85
	50	2.00	1.41	88	1.00	0.00	92
CTAB	0 *	12.5	6.36	0	11.00	5.66	0
	2	13.5	6.36	-8	10.00	4.24	9
	5	10.5	4.95	16	6.50	3.54	41
	10	7.50	3.54	40	5.50	2.12	50
	20	6.50	3.54	48	4.50	2.12	59
	30	3.50	2.12	72	3.00	1.41	73
	50	3.50	2.12	72	2.00	1.41	82
Glutaraldehyde	0 *	11.50	7.78	0	9.50	6.36	0
	2	10.50	7.78	9	7.50	4.95	21
	5	10.00	7.07	13	5.50	3.54	42
	10	8.50	6.36	26	4.50	2.12	53
	20	6.00	5.66	48	3.00	2.83	68
	30	4.00	2.83	65	2.50	2.12	74
	50	2.00	1.41	83	2.00	1.41	79

* control sample

Table 5.7: Biocide concentrations, Specific oxygen uptake rate (SOUR), Standard deviation (SD) and Inhibition percentage (mean values) measured after both 30 minutes and 3 h of biocide exposure in the Arentim WWTP activated-sludge samples (two independent replicates assays)

Biocide	Concentration (mg/g MLSS)	After 30 min			After 3 h		
		SOUR (mg/g MLVSS.h)	SD	Inhibition (%)	SOUR (mg/g MLVSS.h)	SD	Inhibition (%)
Triclosan	0 *	14.50	0.71	0	13.50	0.71	0
	2	15.50	0.71	-7	12.00	0.00	11
	5	8.00	0.00	45	7.50	0.71	44
	10	6.50	0.71	55	5.00	0.00	63
	20	5.00	0.00	66	4.00	0.00	70
	30	4.00	1.41	72	2.00	0.00	85
	50	2.50	0.71	83	1.00	0.00	93
CTAB	0 *	16.50	2.12	0	15.50	2.12	0
	2	20.00	1.41	-21	14.00	1.41	10
	5	13.00	1.41	21	11.50	0.71	26
	10	12.50	2.12	24	11.00	1.41	29
	20	11.00	1.41	33	9.50	0.71	39
	30	10.00	1.41	39	5.50	0.71	65
	50	8.50	0.71	48	3.50	0.71	77
Glutaraldehyde	0 *	18.00	1.41	0	17.5	0.71	0
	2	15.50	3.54	14	13.5	3.54	23
	5	12.00	2.83	33	10.00	0.00	43
	10	11.00	1.41	39	9.00	0.00	49
	20	9.50	2.12	47	7.00	0.00	60
	30	7.00	1.41	61	5.00	0.00	71
	50	4.50	0.71	75	3.00	0.00	83

* control sample

In general, by analysing the three tables, it is possible to verify that Braga WWTP generally presented, after 30 minutes and 3 h of biocide exposure, higher SOUR values compared to the other two WWTP, Celeirós and Arentim.

Braga WWTP processes an urban effluent and presented on average a higher initial SBI (9, class I) comparing to Celeirós WWTP (SBI = 6, class II) which deals with an industrial effluent.

In turn, Arentim WWTP (SBI = 7, class II) despite dealing with an urban effluent, like Braga WWTP, displays an initial SBI similar to the Celeirós WWTP, corresponding to a biological activity in decline, which may have led to lower respiration rates, suggesting a low microbial community health condition. Although Arentim WWTP and Celeirós WWTP presented the same SBI class, this was not reflected into the SOUR values, since those of Arentim WWTP were often superior to Celeirós WWTP values. Regarding the statistical analysis, the respiration rate data showed a good reproducibility with low SD values, being the highest values obtained at Celeirós WWTP and the lowest at Arentim WWTP.

The toxicity evaluated by respirometric sludge respiration rate represents the inhibition of microorganisms in the activated-sludge suspension, since that OUR and SOUR response decreases when wastewater

contains toxicants or inhibitors (Orhon *et al.* 2009). The inhibitory effect of biocides tested (percentage inhibition) at each concentration was calculated from the SOUR values and corresponding controls for Braga, Celeirós and Arentim WWTP, respectively (Figure 5.4, Figure 5.5 and Figure 5.6). Regarding the results for Braga WWTP, all biocides (Figure 5.4 (a), (b) and (c)) showed a dose-dependent effect on biomass respiration inhibition.

Among the tested biocides, triclosan (Figure 5.4 (a)) was the most toxic agent in Braga activated-sludge, since it was the one that after 30 min of incubation lead to higher percentages of inhibition for the same concentration range. Inhibition increased with increasing concentrations of triclosan, in a dose-dependent mode of action (IC_{50} 30 min = 15 mg/g MLSS; 52 µmol/g MLSS and IC_{50} 3 h = 9 mg/g MLSS; 31 µmol/g MLSS). A stimulatory effect in the first 30 min of treatment was observed, upon the addition of biocide (2 mg/g MLSS of triclosan). Comparing the inhibition between controls *vs* biocide concentration, statistically significant differences were found at concentrations of 2 mg/g MLSS ($p \leq 0.05$) and above ($p \leq 0.01$ and $p \leq 0.001$). Overall, the biocide concentration accounted for 94.62 % of the total variance (two-way Anova; $p \leq 0.0001$). The slight differences observed between time points (30 min and 3 h) were considered statistically significant ($p < 0.0001$), which indicates an action of the biocide along time.

CTAB (Figure 5.4 (b)) was the less toxic agent at the selected range if considering mass concentration but the intermediate when considering molar concentration (IC_{50} 30 min = 40 mg/g MLSS; 110 µmol/g MLSS and IC_{50} 3 h = 13 mg/g MLSS; 36 µmol/g MLSS). After 30 minutes of treatment with the highest concentration (50 mg/g MLSS) a little more than 50 % oh the community still had the ability to consume oxygen (respiration). After 3 h of treatment the inhibition percentage increases above 50 %, causing an inhibition of the order of 78 %. As reported for triclosan, the same stimulation at 2 mg/g MLSS was found in the first 30 min of treatment upon addition of biocide, suggesting a stimulation of the microbial activity as an attempt to react to the stress situation caused by CTAB addition. Comparing the inhibition between controls *vs* concentration of biocide, statistically differences were found only at concentrations ≥ 5 mg/g MLSS after 3 h of contact time ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$). Overall, the biocide concentration accounted for 72.60 % of the total variance (two-way Anova; $p < 0.0001$). The differences observed between the two-time points were considered once more statistically significant ($p = 0{,}0007$), which indicates an action of the biocide over time.

Inhibition increased with an increasing concentration of glutaraldehyde (Figure 5.4 (c)), in a dose dependent manner (IC_{50} 30 min = 29 mg/g MLSS; 290 µmol/g MLSS and IC_{50} 3 h = 13 mg/g MLSS; 130 µmol/g MLSS). Comparing the inhibition between control *vs* biocide concentration, statistically

differences were found in all concentrations studied at the two end-points ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$). Overall, the biocide concentration accounted for 91.61 % of the total variance (two-way Anova; $p \leq 0.0001$). The slight differences observed between time points were considered statistically significant ($p = 0.0019$), indicating, once again, an action of the biocide over contact time.

Overall, all biocides tested showed a time-dependency for the same concentrations, revealing a similar trend with significant differences found between 30 min and 3 h of treatment. Furthermore, the biocides also exhibited a dose-dependent effect on the biomass respiration inhibition on Braga WWTP. Triclosan was the most toxic, followed by glutaraldehyde and CTAB, when mass concentration is considered. However, with the IC_{50} conversion from mg/g MLSS to µmol/g MLSS, it is noticeable that the toxicity trend is altered: glutaraldehyde turns out to be the weaker biocide, as said above.

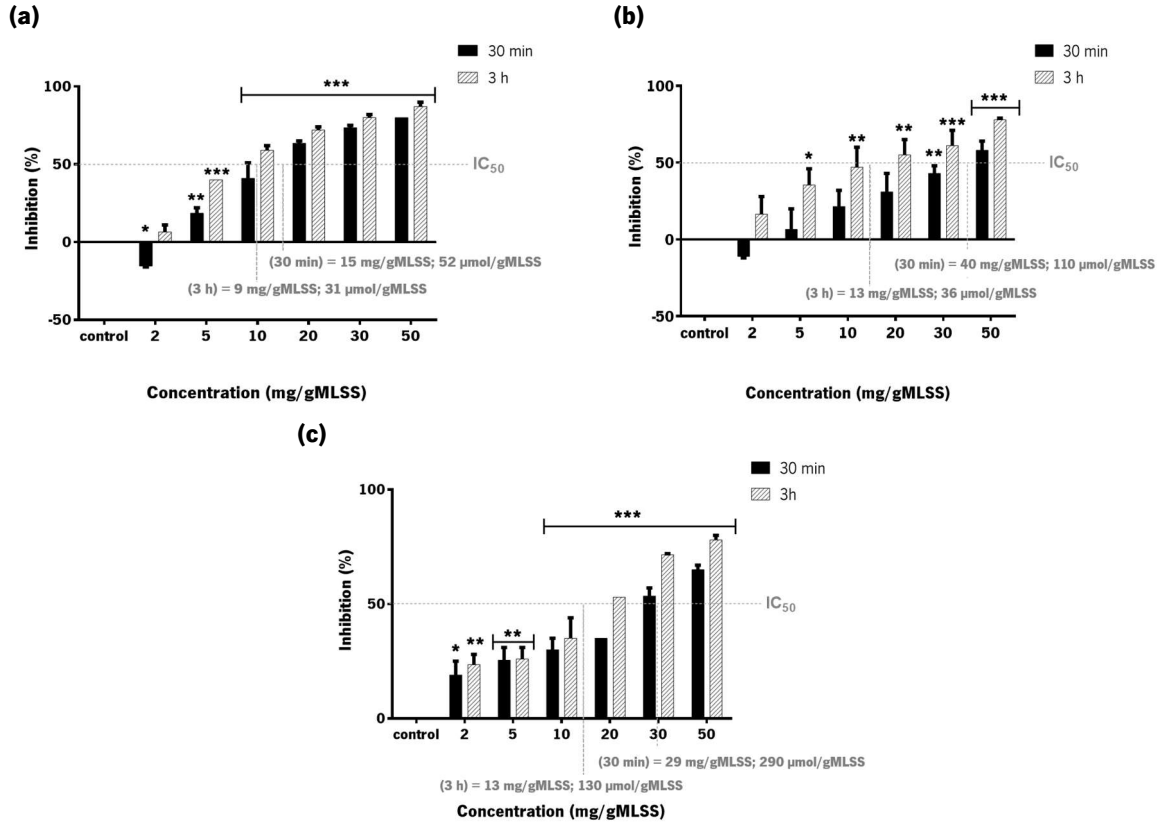

Figure 5.4: Inhibition (%) of activated-sludge from Braga WWTP when exposed to **(a)** triclosan, **(b)** CTAB and **(c)** glutaraldehyde concentrations (mg/g MLSS) in the two end-point assays (30 minutes and 3 h). The IC_{50} value of each biocide after 30 minutes and 3 h is represented by a dashed line. All the columns show the mean ± SEM (n=2). Statistically significant difference with control (0 mg/g MLSS): * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

The inhibition of activated-sludge from Celeirós WWTP increased with increasing concentrations of triclosan, CTAB and glutaraldehyde, as presented in Figure 5.5 (a), (b) and (c). Inhibition percentage increased with increasing concentrations of triclosan (Figure 5.5 (a)), in a dose-dependent manner (IC_{50}

30 min and 3 h = 7 mg/g MLSS; 24 µmol/g MLSS). Once more, triclosan was the most effective biocide, even though the inhibitory effect (behaviour) was roughly generally similar for all biocides. A stimulatory effect a low concentration of biocide (2 mg/g MLSS of triclosan) in the first 30 min of treatment was observed, upon addition of biocide. Comparing the inhibition between controls *vs* biocide concentration, statistically differences were found at concentrations of 2 mg/g MLSS ($p \leq 0.05$) and above ($p \leq 0.001$). Overall, the biocide concentration accounted for 92.63 % of the total variance (two-way Anova; $p \leq 0.0001$). The slight differences observed between 30 min and 3 h of treatment were considered statistically significant ($p = 0.0383$), which indicates an action of triclosan along time.

Concerning CTAB, inhibition of the activated sludge increased with increasing concentrations of the biocide in a dose-dependent manner as displayed in Figure 5.5 (b). In this WWTP, CTAB was once again the less toxic at the selected range of concentrations (IC_{50} 30 min = 25 mg/g MLSS; 69 µmol/g MLSS and IC_{50} 3 h = 19 mg/g MLSS; 52 µmol/g MLSS) if mass concentration is considered. For the highest concentration (50 mg/g MLSS), after 30 min of treatment, the inhibition was already approaching 80 %, significantly higher form the inhibition caused in Braga WWTP. The recurrent stimulatory effect (at 2 mg/g MLSS of CTAB) in the first 30 min of treatment was observed, upon addition of biocide. Comparing the inhibition between controls *vs* concentration of biocide, statistically differences were found at concentrations ≥ 2 mg/g MLSS after 3 h of contact time ($p \leq 0.01$ and $p \leq 0.001$). Overall, the biocide concentration accounted for 96.62 % of the total variance (two-way Anova; $p < 0.0001$). The differences observed between the two-time points were considered once more statistically extremely significant ($p < 0.0001$), which indicates an action of the biocide over time.

Inhibition increased with increasing concentration of glutaraldehyde (Figure 5.5 (c)), in a dose dependent manner (IC_{50} 30 min = 15 mg/g MLSS; 150 µmol/g MLSS and IC_{50} 3 h = 9 mg/g MLSS; 90 µmol/g MLSS). Comparing the inhibition between control *vs* biocide concentration, statistically differences were found at concentrations ≥ 2 mg/g MLSS after 3 h of contact time ($p \leq 0.05$ and $p \leq 0.001$). Overall, the biocide concentration accounted for 92.60 % of the total variance (two-way Anova; $p \leq 0.0001$). The slight differences observed between time points were considered statistically significant ($p = 0.0033$), indicating an action of the biocide over contact time.

Overall, all biocides tested showed a time-dependence for the same concentrations, exhibiting a similar trend with significant differences at 30 min and 3 h of treatment. Moreover, the biocides also exhibited a dose-dependent effect on biomass respiration inhibition on Celeirós activated-sludge. Triclosan was the most effective biocide, followed by glutaraldehyde, and CTAB. However, with the IC_{50} conversion from

mg/g MLSS to µmol/g MLSS it is noticeable that the toxicity trend is altered: glutaraldehyde turns out also to be the less effective biocide, similarly to what was observed in the experiment with Braga activated sludge.

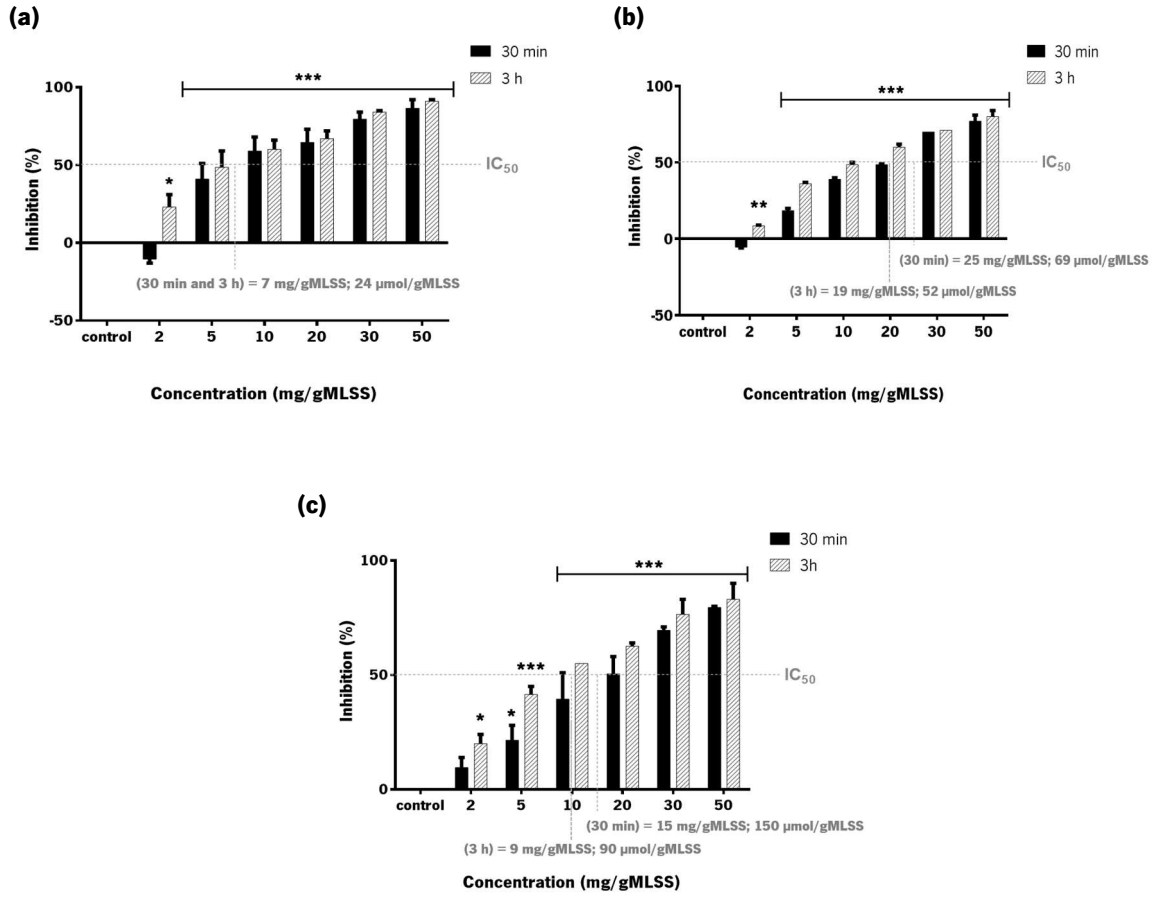

Figure 5.5: Inhibition (%) of activated-sludge from Celeirós WWTP when exposed to **(a)** triclosan, **(b)** CTAB and **(c)** glutaraldehyde concentrations (mg/g MLSS) in the two end-point assays (30 minutes and 3 h). The IC_{50} value of each biocide after 30 minutes and 3 h is represented by a dashed line. All the columns show the mean ± SEM (n=2). Statistically significant difference with control (0 mg/g MLSS): * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

In the case of Arentim WWTP, and similarly to what happened above, triclosan (Figure 5.6 (a)) was the most toxic compound, since it was the one that after 30 min of incubation obtained higher percentages of inhibition for the same concentration range (IC_{50} 30 min and 3 h = 7 mg/g MLSS; 24 µmol/g MLSS). Again after 30 min of treatment, 2 mg/g MLSS concentration exhibited a stimulatory effect. Comparing the inhibition between controls *vs* biocide concentration, statistically differences were found only after 3 h of treatment, at concentrations of 2 mg/g MLSS ($p \leq 0.01$) and above ($p \leq 0.001$). Overall, the biocide concentration accounted for 97.65 % of the total variance (two-way Anova; $p \leq 0.0001$). The differences observed between 30 min and 3 h of treatment were considered statistically significant ($p < 0.0001$), which indicates an action of the biocide along time.

CTAB was also the less effective biocide at the selected range of concentrations studied, as presented in Figure 5.6 (b). After 30 minutes of treatment with the highest concentration (50 mg/g MLSS) only 50 % (49 %) of microorganisms were consuming oxygen (respiration). After 3 h of contact time, the inhibition percentage increases to over 80 %. Therefore, due to the fact that after 30 min of exposure at the highest concentration the inhibition percentage was already at 49 %, it was not possible to calculate the IC_{50}. For the 3 h of exposure, an IC_{50} = 26 mg/g MLSS; 71 µmol/g MLSS was obtained. As reported in triclosan, the stimulation at 2 mg/g MLSS was found in the first 30 min of treatment. Comparing the inhibition between controls *vs* concentration of biocide, statistically differences were in all concentrations studied at the two end-points ($p \leq 0.05$ and $p \leq 0.001$). Overall, the biocide concentration accounted for 85.63 % of the total variance (two-way Anova; $p < 0.0001$). The differences observed between the two-time points were considered once more statistically significant ($p < 0.0001$), which indicates an action of the biocide over time.

Inhibition increased with increasing concentration of glutaraldehyde (Figure 5.6 (c)), in a dose dependent manner (IC_{50} 30 min = 15 mg/g MLSS; 150 µmol/g MLSS and IC_{50} 3 h = 9 mg/g MLSS; 90 µmol/g MLSS). Comparing the inhibition between control *vs* biocide concentration, statistically differences were found at concentrations ≥ 2 mg/g MLSS after 3 h of contact time ($p \leq 0.01$ and $p \leq 0.001$). Overall, the biocide concentration accounted for 93.80 % of the total variance (two-way Anova; $p \leq 0.0001$). The differences observed between time points (30 min and 3 h) were considered statistically significant ($p = 0.0056$), indicating an action of the biocide along contact time.

Overall, all the tested biocides showed a time-dependency for the same concentrations, revealing a similar trend with significant differences found between at 30 min and 3 h of treatment. Furthermore, the biocides also exhibited a dose-dependent effect on biomass respiration inhibition on Arentim WWTP. As in the other two experiments, triclosan was the most effective biocide, followed by glutaraldehyde, and CTAB. And in this case also, with the IC_{50} conversion from mg/g MLSS to µmol/g MLSS, it is noticeable that the toxicity trend is altered: glutaraldehyde turns out to be the less effective biocide.

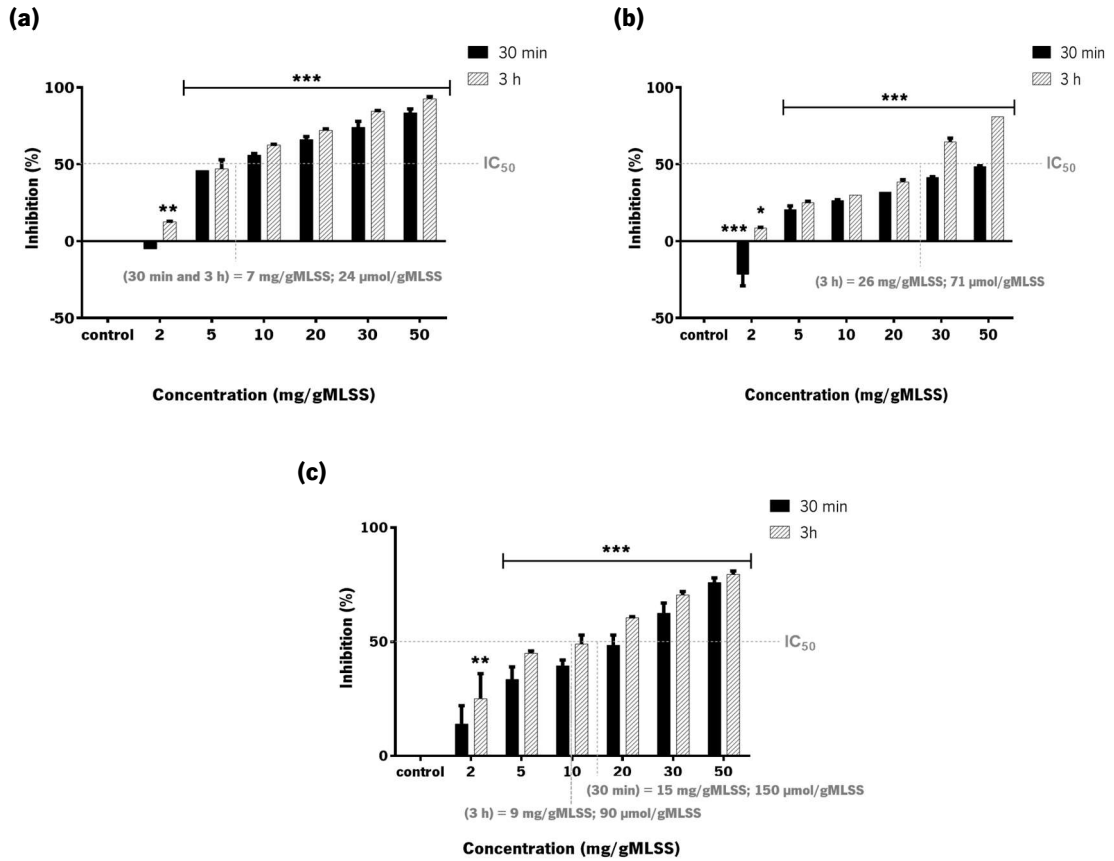

Figure 5.6: Inhibition (%) of activated-sludge from Arentim WWTP when exposed to **(a)** triclosan, **(b)** CTAB and **(c)** glutaraldehyde concentrations (mg/g MLSS) in the two end-point assays (30 minutes and 3 h). The IC_{50} value of each biocide after 30 minutes and 3 h is represented by a dashed line. All the columns show the mean ± SEM (n=2). Statistically significant difference with control (0 mg/g MLSS): * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

The control values for each WWTP varied between the biocides studied, due to the activity variation of the different sludge samples used throughout the respirometry assay. Anyway, for all WWTP, an increasing concentration of triclosan, CTAB and glutaraldehyde showed a gradual decline in SOUR measurements, shortly after 30 minutes of exposure to the biocides. This decrease is even more evident in 3 h of exposure, as expected, which indicate a decay or inhibition of the microorganisms in the sludge. The obtained results in the different assays showed that the biocide concentration and the effect of the biocide had a dose-dependent manner in all WWTP (except at the lowest concentration of triclosan and CTAB), obtaining a higher percentage of inhibition when higher concentration of the biocide was employed. In all WWTP studied, a stimulatory effect was recurrent at the 2 mg/g MLSS concentration for triclosan and CTAB, contrary to the expected inhibition caused by the entry of the toxicants, when compared to the control of the assay. This stimulatory effect of lower concentrations can be explained by the microbial activity, which upon receiving the shock of the toxic (Arai *et al.* 2009), tends to increase the metabolism during the first 30 minutes, and consequently lead to higher absorption of DO, in an attempt to react to the stress situation caused by the biocide addition. After 3 h of contact time, it was possible to verify that

there was no stimulation effect by the biocides, causing only the expected gradual decay or inhibition of the microorganisms with the increased concentration studied.

Considering the three WWTP, the respirometry assay showed that all biocides had a substantial effect on the respiratory capacity of the microorganisms present in activated-sludges, with the highest effect of inhibition belonging to triclosan, followed by glutaraldehyde and CTAB by this order if considering concentration, and CTAB and glutaraldehyde by this order, if considering molar concentrations. The percentages (average values) obtained after the addition of the three biocides in all WWTP, at a concentration of 50 mg/g MLSS were 87 %, 77 % and 71 % inhibition for triclosan, glutaraldehyde and CTAB, respectively. The behaviour of the biocides along the two end-points, 30 min and 3 h of treatment, were more similar between Braga WWTP and Arentim WWTP, corroborating the values obtained for the SOUR. A possible explanation for this occurrence is the fact that the microbial composition in these two WWTP could be more similar among them than between each of the and Celeirós WWTP, because their effluents were more identical to each other than the effluent from Celeirós WWTP, which has an industrial load in the sewage.

5.3.2 Bench-scale assay

5.3.2.1 Activated-sludge performance evaluation

Along with the establishment of the effectiveness of the three biocides - triclosan, CTAB and glutaraldehyde - on the control of filamentous bacteria, it must be ascertained if the biocides do not hamper the performance of the other microorganisms present in the activated-sludge.

WWTP performance is determined by the direct evaluation of the influent and effluent contents and the measured removal efficiency (Puig *et al.* 2010). Routine microbiological analyses provide information about WWTP environmental parameters that are directly correlated to WWTP performance. The presence of biocides or other toxic substances in the influent negatively influence the microbial community, leading to an inefficient WWTP operation (Bodík *et al.* 2008) on one hand, and to microbiological results that reflect this condition.

All assays took 72 h (sampling at 0 h, 24 h, 48 h and 72 h, with 0 h sampling actually carried out after 30 minutes of contact (maximum)). The dominant filamentous bacteria were identified by morphology observation using a microscope and staining reactions (Figure 5.7) - Gram (a) and Neisser (b) - according

to the Eikelboom identification key (Eikelboom 2000). During all the assays, the predominant filamentous bacteria was Eikelboom Type 0092 (Gram negative and Neisser positive - black arrow) and the secondary was Type 0041/0675 (Gram positive and Neisser negative - white arrow). The same pattern of dominant filamentous bacteria was found in both untreated (control) and treated activated-sludge units. Coincidently, these are also filamentous morphotypes that were identified as the most frequent and the most abundant in the initial study of prevalence of filamentous bacteria in Portugal.

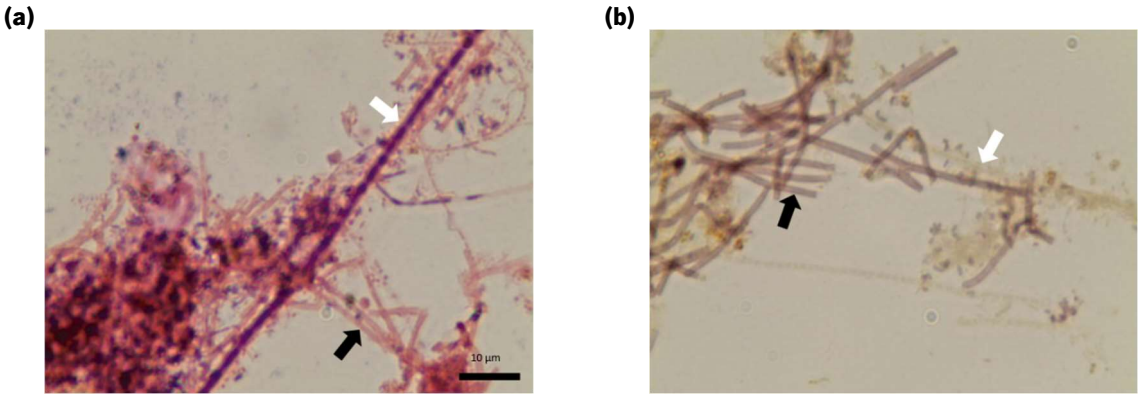

Figure 5.7: Bright-field images of bench-scale assay from control unit (0 mg/g MLSS) at the beginning of treatment (0 h). Gram **(a)** and Neisser **(b)** staining showing Type 0092 (black arrow) and Type 0041/0675 (white arrow), with the magnification of 1000 X (scale bars = 10 µm) for all images

Activated-sludge performance and microbial community were assessed after treatment of three biocides - triclosan, CTAB and glutaraldehyde. The variation profiles of performance parameters (MLSS (mg/L), MLVSS (mg/L), pH, SBI, and filament abundance) for triclosan, CTAB, and glutaraldehyde are shown in Figure 5.8, Figure 5.9 and Figure 5.10, respectively.

Both MLSS (mg/L) (Figure 5.8 (a)) and MLVSS (mg/L) (Figure 5.8 (b)) parameters showed a similar behaviour. At the beginning of the treatment (0 h), solids were always higher than after 72 h, even in control conditions because the conditions of feed, aerating and space limit the biomass growth. When triclosan was added to the sample, the biocide reacted with the activated-sludge and gave rise to the formation of foam. After 72 h of treatment, MLSS and MLVSS values were not significantly different from those of the control unit. However, at this incubation time, the flocs were less dense and more fragmented (Figure 5.11).

The pH values for the control unit decreased gradually from neutral (7.0) to acidic (5.0) throughout the 72 h of the assay. On the contrary, an increase of the pH values from 7.0 to 8.5 was observed for all triclosan concentrations (Figure 5.8 (c)). Acidification of the activated-sludge unit can be justified by the

metabolic activity of the microbial community, which leads to the production of carbon dioxide (CO_2) (Greenwood & Earnshaw 2012; Thauer 1988). The addition of the biocide could disturb the microbial community metabolism, resulting in the alkalization of the environment due to cell lysis (Vandamme 1997).

Regarding the SBI (Figure 5.8 (d)), the highest values were found in the control unit (SBI = 9). At the starting time of the assay (0 h) decreasing values of SBI values were observed with increasing concentrations of triclosan (SBI = 8 at 2 mg/g MLSS to SBI = 7 above 20 mg/g MLSS). This indicates that the addition of triclosan had a biocidal effect on protozoa and metazoan communities. Above 20 mg/g MLSS, SBI values remained constant at the value of 7 (class II) until the end of the assay. Despite of the decay in the biological activity, according to Madoni (1994), class II is still an indicator of good performance of the activated-sludge unit, with a well colonized and stable sludge.

The abundance of all filamentous bacteria was measured by the scoring method of (Jenkins *et al.* 2004) - Figure 5.8 (e) and Figure 5.8 (f). At the starting time (0 h) and at 24 h of incubation, the filament abundance of Type 0092 was considered “score 5” (abundant) in the control unit and this was maintained during the 72 h assay. The same value was found in the units with concentrations of triclosan up to 10 mg/g MLSS after 24 h of biocide treatment. After 48 h of treatment with 5 mg/g MLSS triclosan and higher, the Type 0092 abundance decreased to “score 4” (very common). Despite a clear effect on the filament abundance level upon addition of the biocide, according to Jenkins *et al.* (2004), “score 4” is still characterized by an excess of filamentous bacteria in the activated-sludge units. Regarding Type 0041/0675 (the second most dominant filamentous bacteria), the biocide effect was similar to Type 0092, although the highest initial level of this filamentous abundance was scored “4”. After 48 h of treatment, at concentrations above 5 mg/g MLSS of triclosan, the filament abundance decreased to “score 3” (common) and remained constant until the end of the assay.

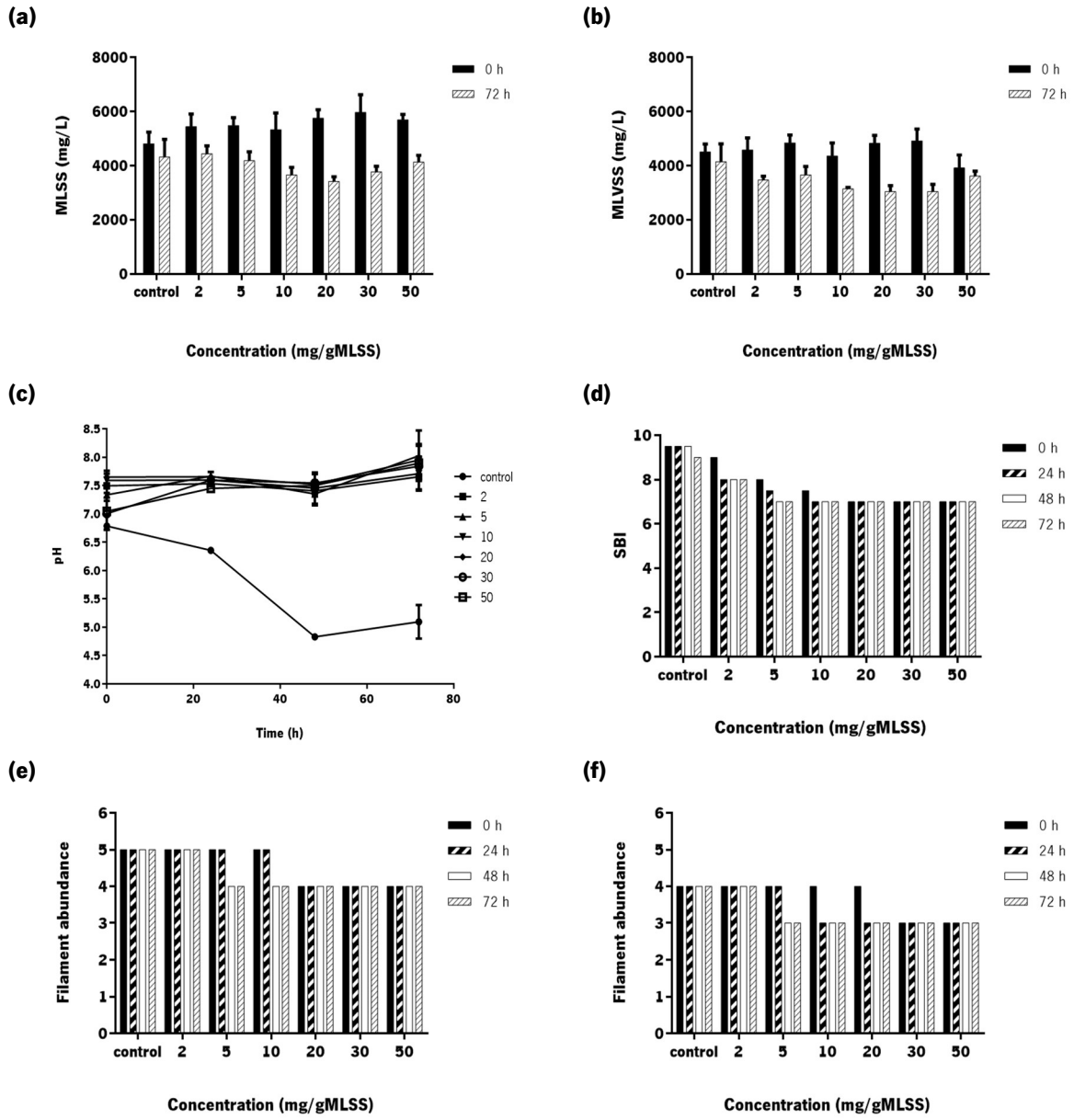

Figure 5.8: Triclosan assay. Variations of **(a)** MLSS (mg/L), **(b)** MLVSS (mg/L), **(c)** pH, **(d)** SBI**, (e)** Type 0092 filament abundance and **(f)** Type 0041/0675 filament abundance, for the control (0 mg/g MLSS) and 2, 5, 10, 20, 30 e 50 mg/g MLSS of triclosan. Plots from **(a)** to **(c)** show the mean ± SEM (n=2).

MLSS (mg/L) (Figure 5.9 (a)) showed similar or slightly non-significant lower values between the starting time (0 h) and the 72 h treatment, even in the cases of exposure to CTAB. This means that CTAB did not significantly altered the biomass maintenance in the reactors. Even so, after 72 h of treatment, the flocs were less dense, more fragmented, despite the similar MLSS and MLVSS concentration (Figure 5.12).

As expected, the pH values for control unit decreased gradually from neutral (7.0) to acidic (5.5) during the 72 h of the assay in control conditions (Figure 5.9 (c)). The concentrations of 2, 5 and 10 mg/g MLSS led to similar profiles, which may point out to a slight to no influence of the CTAB on the overall microbial community. CTAB concentrations of 20 and 30 mg/g MLSS exhibited a pH profile slightly higher than the control unit. The concentration of 50 mg/g MLSS displayed a constant alkaline profile with values ranging approximately between 7.5 and 8.0. Concerning the pH profile, only the highest CTAB concentration exhibited a clear negative effect on the microbial community metabolism.

Regarding the SBI (Figure 5.9 (d)), no difference was found between the control unit (SBI = 9) and CTAB lowest concentrations (2 and 5 mg/g MLSS), remaining both constant until end of assay. The 10 mg/g MLSS CTAB concentration only led to a decrease in the SBI value after 72 h of treatment. The CTAB concentrations of 20 and 30 mg/g MLSS caused a decrease in the SBI value after 24 h to 7 (class II) at 48 h and 24 h of treatment, respectively. The SBI of the activated-sludge unit treated with 30 mg/g of CTAB, MLSS dropped to 6 at 72 h. In the concentration of 50 mg/g of CTAB, MLSS suffered the steepest decrease, SBI reaching the value of 2 after 72 h of treatment. The value 2 (class IV) of the SBI in the highest CTAB concentration was due to a total dominance of *Tetrahymena pyriformis* (ciliate swimmer) in the treated activated-sludge unit; its presence is commonly associated with excessive loads and the quality of the effluent produced is generally poor, corresponding to a low performance (Madoni 1994). This indicates that the addition of CTAB had a clear biocidal effect in protozoa and metazoan community in the highest concentrations.

Throughout the duration of the assay, the filament abundance of Type 0092 (Figure 5.9 (e)) was considered “score 4” (very common) in the control unit and in all CTAB concentrations below 50 mg/g MLSS. The same value was found for 50 mg/g MLSS CTAB concentration until 24 h of treatment, after which the filament abundance decreased to “score 3” (common) and remained constant until the end of the assay. CTAB showed a positive effect against filamentous bacteria only at the highest CTAB concentration, since it reduced the “score” value from 4 to 3. Regarding Type 0041/0675, the score value remained constant throughout the assay, scored 4 in the control unit, and 3 in all CTAB

concentrations (Figure 5.9 (f)). CTAB apparently exhibited a reduction in the "score" level that is independent from its concentration and incubation period (0 h, 24 h, 48 h and 72h).

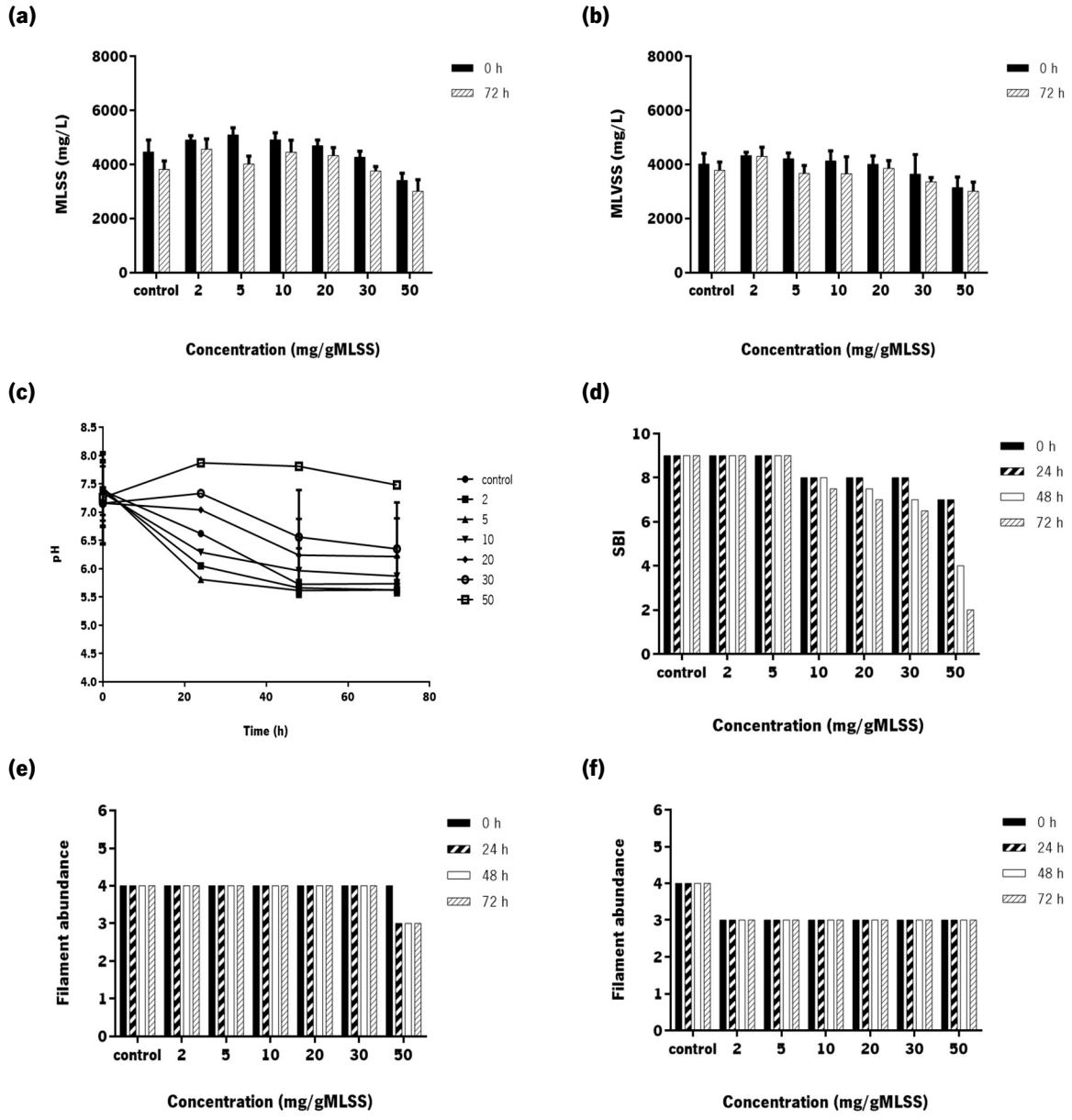

Figure 5.9: CTAB assay. Variations of **(a)** MLSS (mg/L), **(b)** MLVSS (mg/L), **(c)** pH, **(d)** SBI**, (e)** Type 0092 filament abundance and **(f)** Type 0041/0675 filament abundance, for the control (0 mg/g MLSS) and 2, 5, 10, 20, 30 e 50 mg/g MLSS of CTAB. Plots from **(a)** to **(c)** show the mean ± SEM (n=2).

MLSS (mg/L) (Figure 5.10 (a)) displayed a similar behaviour in all tested concentrations, including the control as well as the MLVSS (mg/L) (Figure 5.10 (b)) with non-significant differences among them. With the increasing glutaraldehyde concentrations, it was clearly observable through microscopy that the flocs were less dense and more fragmented (Figure 5.13).

The pH values for the control unit decreased gradually from neutral (7.0) to acidic (5.5) during the 72 h of the assay (Figure 5.10 (c)). The concentrations of 2, 5, 10 and 20 mg/g MLSS showed a similar profile. The biocide effect was notorious only after 24 h of treatment or at the highest glutaraldehyde concentrations, namely 30 and 50 mg/g MLSS, with values ranging approximately between 7.3 - 6.4 and 7.3 - 7.1, respectively.

Regarding the SBI (Figure 5.10 (d)), the highest value observed was 9, found in the control unit and with glutaraldehyde concentrations of 2 and 5 mg/g MLSS. All remained constant until the end of the assay. At the starting time of the assay (0 h), decreasing values of SBI values were observed with increasing concentrations of glutaraldehyde (SBI = 8 at 10 to 30 mg/g MLSS, to SBI = 7 at 50 mg/g MLSS). This indicates that the addition of glutaraldehyde had a biocidal effect in protozoa and metazoan communities. Above 10 mg/g MLSS concentration, SBI values started to decrease successively after 24 h of treatment to values of 7 (class II) for 10 and 20 mg/g MLSS and 6 (class II) for 30 and 50 mg/g MLSS. Despite of the decay in the biological activity, according to Madoni (1994), class II is still an indicator of good performance of the activated-sludge unit, with a well colonized and stable sludge.

The filament abundance of Type 0092 (Figure 5.10 (e)) was considered “score 5” (abundant) in the control unit and no changes were observed with concentrations up to 5 mg/g MLSS glutaraldehyde. For higher concentrations, the filament abundance decreased to “score 4” (very common) until the end of the assay. Type 0041/0675 (the second more dominant filamentous bacteria), the biocide effect was similar to Type 0092, although the highest level in the control unit was “score 4” (Figure 5.10 (f)). During the assay, the filament abundance decreased throughout the incubation period (“score 3” - common) with all glutaraldehyde concentrations. Glutaraldehyde apparently led a reduction in the “score” level that is independent from its concentration and incubation period (0 h, 24 h, 48 h and 72h).

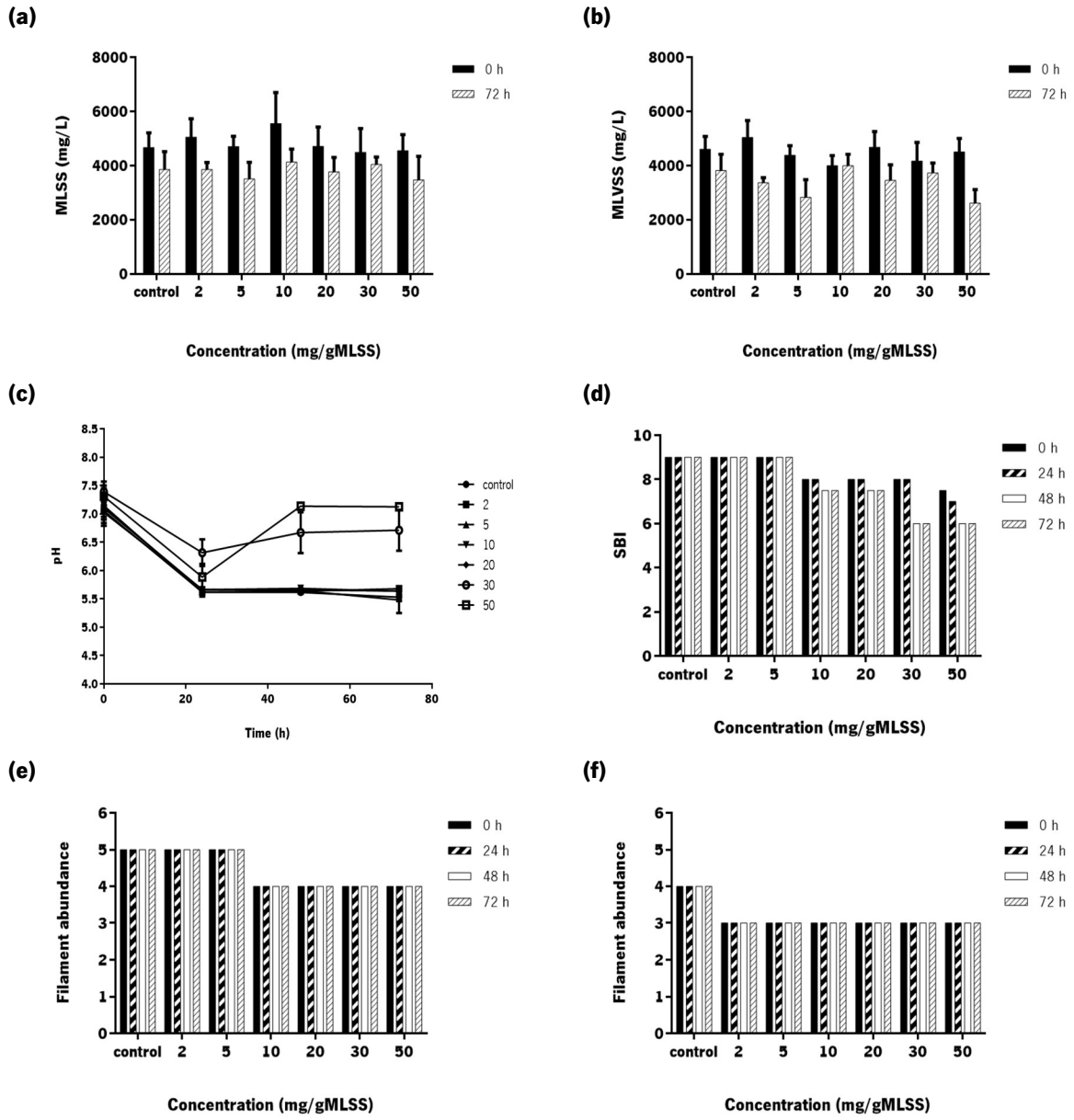

Figure 5.10: Glutaraldehyde assay. Variations of **(a)** MLSS (mg/L), **(b)** MLVSS (mg/L), **(c)** pH, **(d)** SBI**, (e)** Type 0092 filament abundance and **(f)** Type 0041/0675 filament abundance, for the control (0 mg/g MLSS) and 2, 5, 10, 20, 30 e 50 mg/g MLSS of glutaraldehyde. Plots from **(a)** to **(c)** show the mean ± SEM (n=2).

Overall, the three tested biocides, during the 72 hours of treatment showed a clear biocidal effect at the used concentrations. The toxic effect of triclosan, both in the microbial community and in the performance of activated-sludge units, was noticeable only after 24 hours, except in the case of the highest concentrations, with the exception of the pH profile assessment which exhibited the toxic affect soon after the adding of the biocide. CTAB caused the weakest effects on what performance and health state of the various communities is respected, with the exception of the highest concentration that caused both a clear and sudden change in the pH profile and in the SBI value. Glutaraldehyde led to intermediate responses, with the highest concentrations leading to significant alterations both in the pH profile and SBI values but the lowest ranging from no alterations to slight or delayed effects.

The present study also intended to verify if the biocides addition in the activated-sludge units could be used as a measure of filamentous bulking control. In the case of Type 0092 (Gram negative), CTAB addition allowed a positive effect on reducing its abundance only at the highest concentration (50 mg/g MLSS), triclosan caused reduction of these filaments straight after the adding only for concentrations higher than 20 mg/g MLSS and for the concentrations of 5 and 10 mg/g MLSS after a delay of at least 24 h and glutaraldehyde caused a lethal immediate effect since the concentration of 10 mg/g MLSS. Considering Type 0041 (Gram positive), CTAB and glutaraldehyde caused a rapid decrease of one score unit with all concentrations, but triclosan had no effect in the lowest concentration (2 mg/g MLSS), a delayed effect with the intermediate concentrations and only the highest concentrations caused an instantaneous decrease of one score unit. These observations, however, must be taken carefully, as the initial scorings were often different and, for this, the definitive limiting effect cannot be strictly established. Interestingly, the Gram Negative Type 0092 seems to be more resistant than the Gram Positive Type 0041/0675, namely in the cases of CTAB and glutaraldehyde, this time partially supporting data from other works (Johnson et al. 2002; Wales & Davies 2015). The biocidal activity of triclosan was similar for both filaments.

5.3.2.2 Live/Dead viability assay (Epifluorescence microscopy)

Cell viability assessment is crucial for the evaluation of cellular health when exposed to toxic compounds, and it can be assessed by morphological modifications, changes in membrane permeability and/or physiological state inferred from the exclusion of certain dyes or the capture and retention of others (Johnson *et al.* 2013; Rampersad 2002). The advantage of the live/dead staining procedure applied in this work was that the respective green and red fluorescence of SYTO-BC and PI were easily distinctive

by fluorescence microscopy. Bearing this in mind, it was expected to clearly visualize the population of live *vs* dead microbial community.

Samples were taken from activate-sludge units and were stained according to the methodology described in section 5.2.6. Fluorescence microscope images of viable and non-viable cells stained with SYTO-BC and PI, respectively, were obtained at the beginning of treatment (0 h) and after 24 h, 48 h and 72 h of treatment for all the biocide concentrations studied.

The effect of biocides on the microbial community was performed through the morphology observation of the live/dead filamentous bacteria by fluorescence microscopy. Among the six initial concentrations (2, 5, 10, 20, 30 and 50 mg/g MLSS), a preliminary assay was conducted to select the concentrations that showed a more evident or visible effect after treatment with biocides. The concentrations of 2 and 5 mg/g MLSS showed no biocidal effect comparing to untreated activated-sludge unit (control). For the concentration of 50 mg/g MLSS, all filamentous bacteria were dead at all end-points. For this reason, the above-mentioned concentrations were excluded and only the intermediate biocide concentrations of 10, 20 and 30 mg/g MLSS were used.

A one-letter code was assigned for the different biocides (T for triclosan, C for CTAB, and G for glutaraldehyde) and the numbers (10, 20 and 30) were used for the biocides concentrations chosen. All the images were taken with low magnification (100 X).

Control units were used to compare the visible effect of biocides on the microbial community for triclosan (Figure 5.11), CTAB (Figure 5.12) and glutaraldehyde (Figure 5.13). For technical reasons referred in section 5.2.3.2, different samples of renewed activated-sludge were used throughout the bench-scale assay and consequently variations in the microbial composition were expected. Variability inherent to the activated-sludge unit was minimised by performing all assays related to each biocide using the same activated-sludge sample. Hence, control units showed characteristic features at different time end-points; at the starting time (0 h) most of live (green) bacteria were in the filamentous form and mainly located in the outer layer of the flocs, most of them dense, well-structured flocs. From 24 h to 72 h of incubation, flocs were gradually less dense, more fragmented, consequence of increasingly dead bacteria.

A dose-dependent effect of triclosan on the microbial community was observed on the activated-sludge unit from 10 to 30 mg/g MLSS (Figure 5.11). At the concentration of 20 mg/g MLSS, flocs were smaller and less dense when compared to the control unit. Therefore, an increasing number of dead cells located

essentially inside the flocs was observable. Besides identification of live/dead fragments of filamentous bacteria, the living cells showed a decrease in the intensity of the green fluorescence. At the concentration of 30 mg/g MLSS most filamentous bacteria were dead (red flocs), with few live (green) fragments of filaments and single cells. Fragmentation of the filamentous bacteria was clearly shown at a higher magnification (1000 X) in Figure 5.14. An immediate effect of triclosan upon addition to the activated-sludge unit can be explained by the fact that triclosan is an oxidizing biocide acting rapidly, either inhibiting microbial growth or eliminating the bacteria (Escalada *et al.* 2005).

After 24 h of triclosan treatment, at a concentration of 10 mg/g MLSS, smaller and less dense flocs were observed comparing with the previous time end-point. Above 20 mg/g MLSS, an increase in the number of dead filamentous bacteria was clearly visible; flocs were mostly stained red. Live green cells were only found in the single cell form or in very small fragments (Figure 5.14). The same effect was observed after 48 h and 72 h of treatment with triclosan.

A dose-dependent effect of CTAB on microbial community of the activated-sludge unit was observed from 10 to 30 mg/g MLSS (Figure 5.12). At 0 h an immediate biocidal effect was visible even at the lowest concentration. The flocs were smaller and less dense comparing to the control unit. Nevertheless, most filamentous bacteria were still viable (green stained) present mainly as single cells. At the highest concentration of 30 mg/g MLSS, the biocidal effect of CTAB was more evident: flocs were orange/red, indicating the presence of injured or dead cells (mostly in single cell form). At higher magnification (1000 X) (Figure 5.14) it was possible to observe that in the same filament some fragments were viable and some fragments were dead.

After 24 h of treatment at 10 mg/g MLSS, smaller and less dense flocs were observed; the fragmentation of live filamentous bacteria was more evident, and most were seen as single cells. Above 20 mg/g MLSS the biocidal effect was more pronounced: the flocs were less structured. In higher magnification (Figure 5.14) intact dead filaments could be observed inside the flocs, whereas the live cells in the outer layer were fragmented, mostly as single cells. The viable filaments, when present, showed a reduction of the green fluorescence intensity comparing to the previous time end-point. The same effect was observed after 48 h and 72 h of treatment.

A dose-dependent effect of glutaraldehyde on the microbial community was observed from 10 to 30 mg/g MLSS (Figure 5.13), even though the effect was not as pronounced as it was with triclosan and CTAB. At the starting-time of the treatment (0 h) no visible differences between the control unit and the lowest

concentration (10 mg/g MLSS) were observed. At the concentration of 20 mg/g MLSS, some fragmentation of the flocs was already observed but most of the filamentous bacteria were still viable. Dead bacteria were observed as single cells. At the highest concentration (30 mg/g MLSS) the biocide effect was more evident: smaller and less dense flocs with dead single cell bacteria, mainly inside the flocs, and reduction of the green fluorescence intensity of both bacteria form filamentous and single cell. This effect is shown at higher magnification (1000 X) in Figure 5.14.

After 24 h of glutaraldehyde treatment, most bacteria were green (live), being also visible fragmented dead cells at the concentration of 10 mg/g MLSS. At the concentration of 20 mg/g MLSS, flocs were more dispersed, mostly consisting of live bacteria. At the highest concentration (30 mg/g MLSS) smaller flocs with fragmented live single cell bacteria in the outer layer of the floc and dead filaments in the inside layer, were observed (Figure 5.14). After 48 h and 72 h of glutaraldehyde treatment, at the lowest concentration, flocs with viable bacteria were still dominant. Above 20 mg/g MLSS, the biocidal effect was more evident: the flocs were increasingly depleted of biomass (losing compactness), with fewer filaments and more single cells. Both CTAB and glutaraldehyde are usually more stable non-oxidizing biocides with longer lasting effects, therefore more slowly inhibiting growth or eliminating the microbial community (Pereira & Vieira 2001; Rochdi *et al.* 2014).

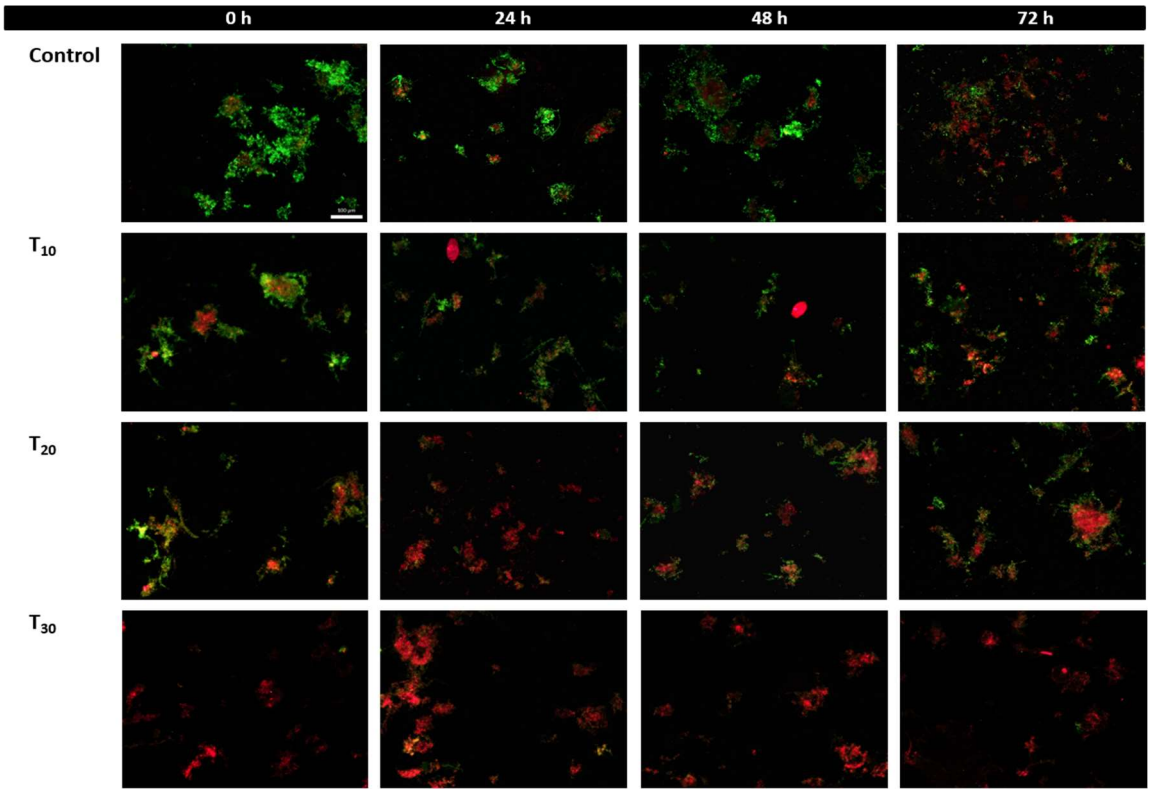

Figure 5.11: Epifluorescence images of bench-scale assay from control unit (0 mg/g MLSS) and 10, 20 and 30 mg/g MLSS concentration of triclosan (T_{10}, T_{20} and T_{30}) at the beginning of treatment (0 h) and after 24 h, 48 h and 72 h of biocide treatment. Live/dead cell viability assay showing live cells stained with SYTO-BC (green) and dead cells stained with PI (red), with the magnification of 100 X (scale bars = 100 µm) for all images.

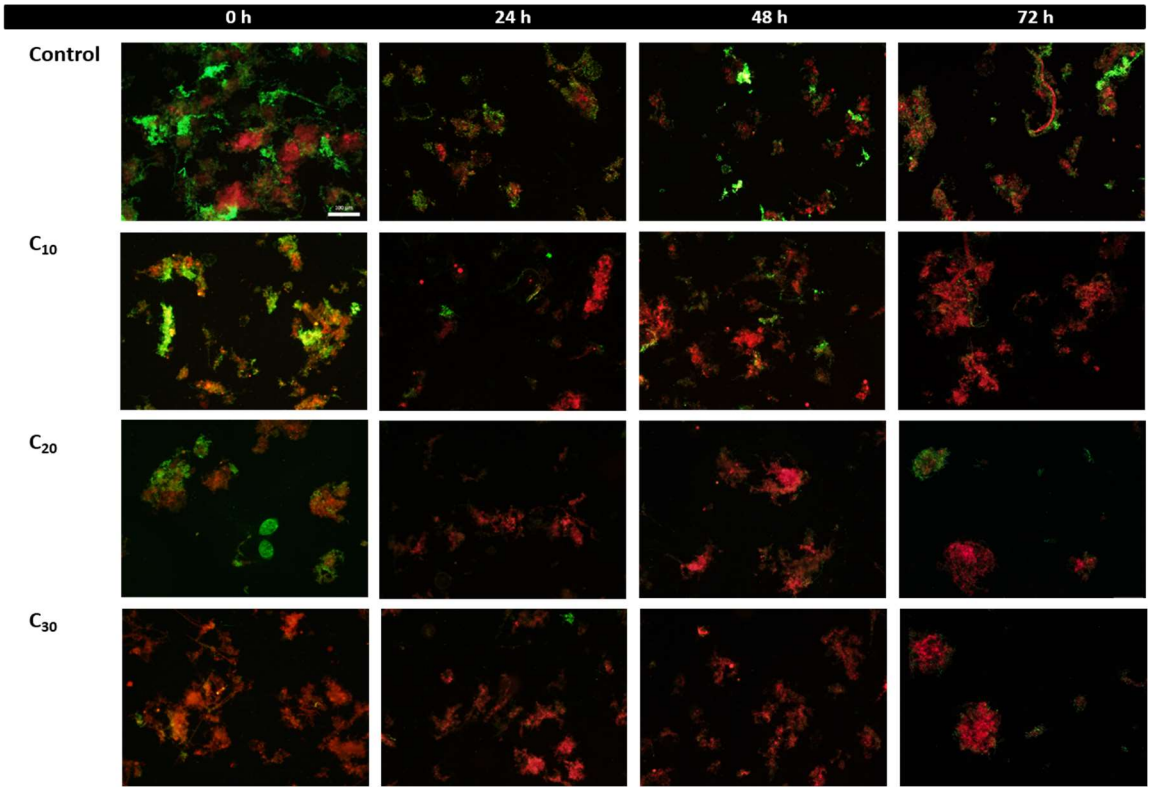

Figure 5.12: Epifluorescence images of bench-scale assay from control unit (0 mg/g MLSS) and 10, 20 and 30 mg/g MLSS concentration of CTAB (C_{10}, C_{20} and C_{30}) at the beginning of treatment (0 h) and after 24 h, 48 h and 72 h of biocide treatment. Live/dead cell viability assay showing live cells stained with SYTO-BC (green) and dead cells stained with PI (red), with the magnification of 100 X (scale bars = 100 µm) for all images.

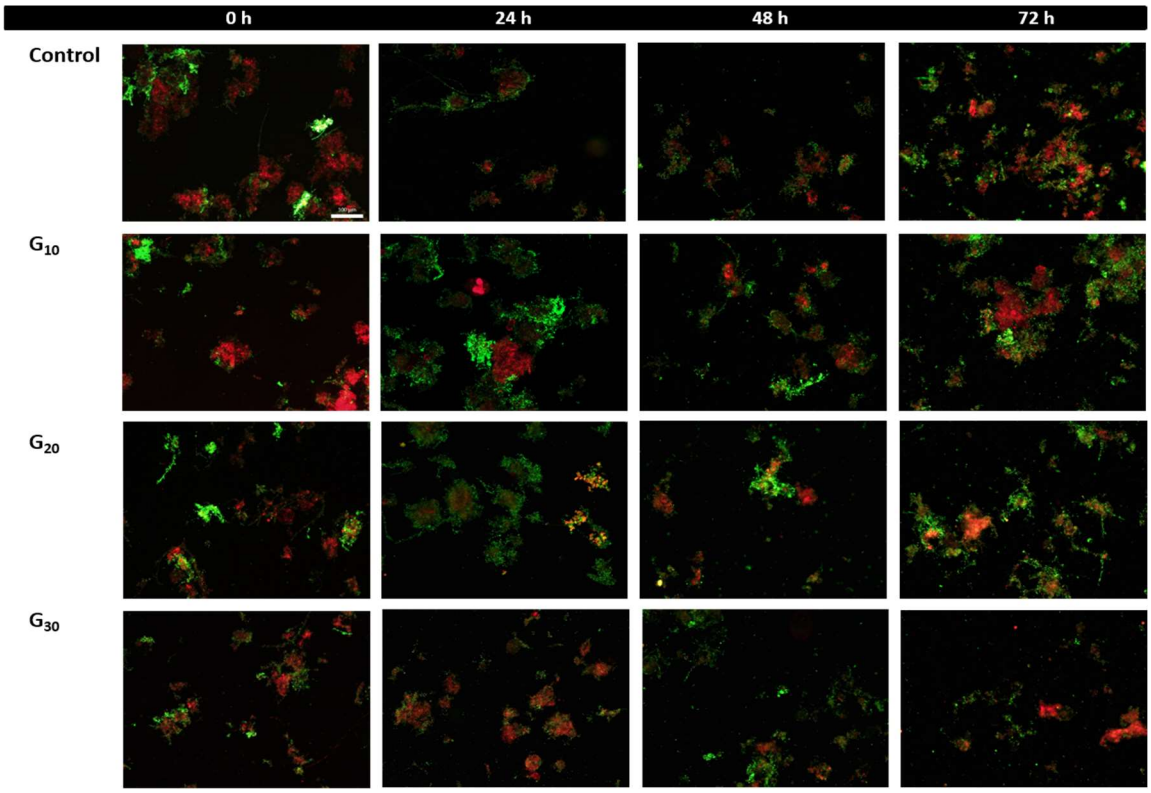

Figure 5.13: Epifluorescence images of bench-scale assay from control unit (0 mg/g MLSS) and 10, 20 and 30 mg/g MLSS concentration of glutaraldehyde (G_{10}, G_{20} and G_{30}) at the beginning of treatment (0 h) and after 24 h, 48 h and 72 h of biocide treatment. Live/dead cell viability assay showing live cells stained with SYTO-BC (green) and dead cells stained with PI (red), with the magnification of 100 X (scale bars = 100 μm) for all images.

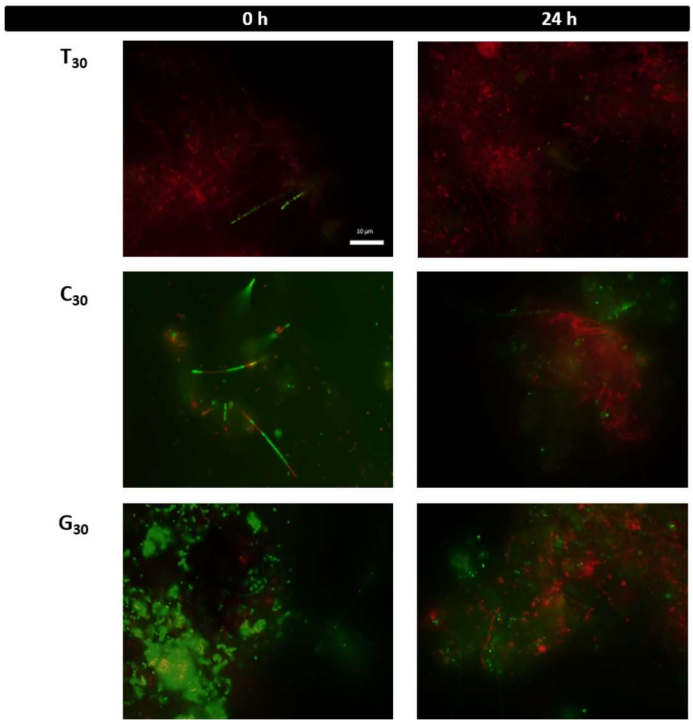

Figure 5.14: Epifluorescence images of bench-scale assay from 30 mg/g MLSS concentration of triclosan, CTAB and glutaraldehyde (T_{30}, C_{30} and G_{30}), respectively, at the beginning of treatment (0 h) and after 24 h of biocide treatment. Live/dead cell viability assay showing live cells stained with SYTO-BC (green) and dead cells stained with PI (red), with the magnification of 1000 X (scale bars = 10 μm) for all images.

Overall, at the starting time (0 h), all biocides (except glutaraldehyde), showed an evident biocidal effect at the concentration of 30 mg/g MLSS (loss of floc structure, fragmentation of filamentous bacteria). This effect was even more pronounced after 24 h of treatment. Among the tested biocides, triclosan and CTAB showed a clear fragmentation effect, mostly in the outer layer of the flocs.

5.4 Conclusions

For all WWTP, an increasing concentration of triclosan, CTAB and glutaraldehyde led to a net decline in SOUR measurements, shortly after 30 minutes of exposure to the biocides. This decrease was more evident after 3 h of exposure, which indicates a decay or inhibition of the microorganisms in the sludge. In the three WWTP, all biocides tested showed a dose-dependent effect in the biomass respiration inhibition. Although a stimulatory effect was observed at low biocide concentrations (2 mg/g MLSS) of triclosan and CTAB, at higher concentrations triclosan was the most toxic and, depending if considering mass concentration or molar concentration, CTAB and glutaraldehyde were the least toxic, respectively.

In the bench-scale assays, all three tested biocides, showed a toxic effect during the 72 hours of treatment, both in the microbial community and in the performance of activated-sludge units, at the used

concentrations. Nevertheless, the effects were more evident in the case of triclosan in what the pH profile and the SBI evaluation is respected and variable in the case of the filamentous control; the effect of CTAB was surprisingly discreet except in the highest concentration (50 mg/g MLSS) or in the assessment of filamentous control of Type 0041/0675. Glutaraldehyde has an intermediate response, between triclosan and CTAB, namely in the control of filamentous Types 0092 and 0041/0675.

It was possible to conclude that, concerning the dominant filamentous bacteria Type 0041/0675 (Gram positive), CTAB and glutaraldehyde exhibited a positive effect in controlling its density independent of concentration and incubation time. Triclosan showed a delay in the efficacy in reducing filaments overgrowth in the intermediate concentrations and no effect in the lowest concentration. The Gram negative Type 0092 showed higher resistance to the biocidal activity in all cases.

The staining with SYTO-BC and PI allowed to easily distinguish the live *vs* dead populations. Loss of floc structure (smaller and with lower density), increase of dead cells and fragmentation of filamentous bacteria to single cells were observed as a result of biocide addition.

It is clear that the effectiveness of biocides, particularly in controlling the growth of filamentous bacteria, must be verified by SVI monitoring, regular analysis of the effluent quality and microscopic observation - as long as that the other components and the general performance are monitored, including the possible effects on the microfauna, because they have different effects on the various components of the communities and on the general performance of the WWTP.

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CHAPTER 6

Conclusions and future perspectives

In this chapter, general conclusions obtained from the present doctoral thesis and future research directions in this field of research are presented. The scientific outputs are also outlined.

6.1 General conclusions

New and emerging pollutants of water systems, resources of increasing need, is a serious problem that has received attention in recent decades. To prevent and minimize the hazardous impacts caused by increasing environmental pollution, urgent measures must be taken to ensure the quality of the disposable water. Over the last decades, various efforts have been made to develop alternative and, particularly, environmentally friendly low-cost technologies for the removal these contaminants because it is of general acceptance that is nowadays imperative that WWTP treat adequately the wastewaters before discharging into the usual receptive water bodies. Bacteria have the fundamental role in the biological treatment for effective removal of the organic pollutants, but the overgrowth of filamentous bacteria endangers WWTP performance. The present research is centred in the assessment of in-use biocides as potential control compounds of the filamentous bacteria populations, but also to clarify the impact of these chemicals in the biological communities of WWTP. First, a survey of the filamentous bacteria in Portuguese WWTP was conducted and the physical-chemical and operational parameters related with their prevalence in the aerated tank were also investigated. Then, the biocide effect of triclosan, CTAB and glutaraldehyde on specific filamentous bacterial populations were studied in *in-vitro* assays. Finally, a study was carried out to evaluate the effect of the same biocides in conditions close to reality using activated-sludge collected from the WWTP, increasing the complexity of the system due to the multiplicity of strains co-existing in a real activated-sludge. The main conclusions that can be withdrawn from the obtained results are summarized below.

The study of the filamentous bacteria identified in Portuguese WWTP (CHAPTER 3) showed that substantial differences were found in frequency, abundance and dominance. The most frequent and abundant morphotypes were Type 0092 and Type 0041/0675 and the most frequently dominant were exactly those that revealed to be problematic: they were the dominant morphotypes during the bulking occurrences in WWTP: Type 1851 and *Microthrix parvicella*. Moreover, study of correlations (Kendall and PCA) between conventional environmental parameters *vs* filamentous bacteria prevalence in aeration tank was verified. When comparing these results with the literature, it is possible to observe that, for the same environmental parameter, there are contradictions among published results, which emphasizes the importance of distinguishing between frequency, abundance and dominance filamentous morphotypes in domestic and industrial WWTP.

In regard of *in-vitro* assays (CHAPTER 4), two filamentous bacteria often present in activated-sludge, *N. amarae* and *S. natans*, were exposed to different concentrations of triclosan, CTAB and glutaraldehyde. These species were selected among the few strains existing in the Collections of Cultures. In these experiments, it was possible to verify a dose-dependent effect on bacterial viability, and a different action mechanism for each biocide. Also, differences in susceptibility to exposure to the tested biocides suggested strain-dependent effects. The range of concentrations when assessing the biocidal effect on *N. amarae* comparative to *S. natans* was about 5-fold to 25-fold higher for glutaraldehyde and triclosan, respectively. CTAB, had a similar biocidal effect on both strains on what dose range is respected. All biocides lead to morphological modification of the filaments leading to their fragmentation to single cells. After 48 h after biocide removal, none of the species had recovered from this biocidal effect. CTAB was found to present the highest biocidal effect among the three used to *N. amarae* and the intermediate toxic when considering the assays with *S. natans*.

In CHAPTER 5, bench-scale assays were conducted to evaluate biocide global effects using samples of real activated-sludge (mimicking WWTP). In a first stage experiments, respirometric assays allowed to infer that all biocides had a significant effect on the microorganisms respiratory capacity, with the highest inhibition percentage belonging to triclosan, followed by glutaraldehyde and CTAB if considering mass concentration and the reverse order (between glutaraldehyde and CTAB) when molar concentration was taken in consideration. In a second stage, the overall performance was assessed and the results allowed to infer the effect of biocides both in the microbial community and in the performance of activated-sludge experimental units along the 72 hours of treatment. A biocidal effect was perceptible for triclosan (biocide with more toxic effects) at the lowest concentrations, and for CTAB and glutaraldehyde the effect was only significant at higher concentrations. Two dominant filamentous bacteria were found: Type 0092 and Type 0041/0675, exactly the two more frequent and abundant in the study described in Chapter 3. The Gram negative Type 0092 revealed to be the most resistant to the biocides, corroborating published works, but contradicting the results obtained in the chapter 4, where the Gram negative *S. natans* revealed to be the most sensitive to the same biocides. It was also possible to infer that the biocides addition gave rise to loss of floc structure (lower density) and to fragmentation of filamentous bacteria into single cells.

Briefly, this work showed that is possible to take advantage of the presence of these biocides, as a non-conventional environmental condition, in the overall performance of activated-sludge processes, particularly in the filamentous bulking control, as long as the other components and overall performance

are monitored and taken into account. It is also showed that each situation must be previously studied as general statements on resistance or susceptibility of certain groups of bacteria to the biocides cannot be made with the present knowledge.

6.2 Future perspectives

The work described in this thesis allowed to conclude that further research with new approaches on this topic could be developed. Some guidelines for future perspectives will be presented.

An interesting option would be to investigate the ability to develop, optimize and perform a lab-scale WWTP prototype being fed with real sewage, in order to better understand the changes in the microbial community and to assess the performance of the treatment system in a more systematic and realistic way. Pre-treated hospital wastewater could be attractive to be used as affluent. Its constitution differs from other effluents and therefore has to be treated differently as it results in significant volumes of wastewater loaded with microorganisms along with heavy metals, toxic chemicals and radioactive elements. On the other hand, such chemicals can have a particular importance in inhibiting or stimulating certain filamentous bacteria types.

It would also be interesting to investigate the action of the triclosan, CTAB and glutaraldehyde in previously installed filamentous bulking and/or foaming initially at lab-scale WWTP. The results at lab-scale would allow to obtain indications concerning operational strategies to be applied to enhance the performance of activated-sludge systems.

Considering the influent entrance in activated-sludge process loaded with biocides in sub-lethal concentrations, other contaminates that have not been considered in this dissertation, such as microplastics, antibiotics, contraceptives and other hormones, have received some attention in recent years. It would also be attractive to investigate the potential control of filamentous bacteria in WWTP in the presence of other contaminants.

6.3 Scientific outputs

The work presented in this doctoral thesis originated scientific outputs in proceedings of scientific conferences and some manuscripts for submission to scientific international journals (already published, submitted or in preparation). The list of the scientific outputs is above listed.

Papers in international scientific periodicals with referees

Liliana Araújo dos Santos, **Vânia Ferreira**, Marta Martins Neto, Maria Alcina Pereira, Manuel Mota, Ana Nicolau, (2015). Study of 16 Portuguese activated sludge systems based on filamentous bacteria populations and their relationships with environmental parameter. *Applied Microbiology and Biotechnology*, **99** (12), 5307-5316.

Papers submitted to publication

Ferreira, V., Dias, N., Mota, M., Pereira, M.O., Nicolau, A., *In-vitro* assessments of biocides effect on specific filamentous bacterial populations. Submitted to publication in *International Journal of Environmental Research and Public Health* (2022).

Ferreira, V., Dias, N., Mota, M., Pereira, M.O., Nicolau, A., Evaluation of biocides action on microbial community and on the performance of activated-sludge. Submitted to publication in *Applied and Environmental Microbiology* (2022).

Communications in scientific meeting

Poster Presentations

Jorge Padrão, **Vânia Ferreira**, Maria S. Duarte, M. Madalena Alves, Ana Nicolau, (2017). Optimizing lab-scale wastewater treatment reactors operation for enhanced assays. *CEB Annual Meeting 2017*, Braga, Portugal, July 06.

Vânia Ferreira, Nicolina Dias, Maria Olívia Pereira, Manuel Mota, Ana Nicolau, (2017). Use of biocides in the control of filamentous bulking in activated-sludge. *CEB Annual Meeting 2017*, Braga, Portugal, July 06.

Vânia Ferreira, Susana Cortez, Jorge Padrão, Daniela P. Mesquita, Nicolina Dias, M. Salomé Duarte, Eugénio C. Ferreira, M. Madalena Alves, Manuel Mota, Ana Nicolau, (2018). Impact of professional cleaning agent used in fish-canning industry in wastewater treatment. *ICOETOX 2018*, Matosinhos, Portugal, October 24-26.