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Negative impacts of cleaning agent DEPTAL MCL® on activated sludge wastewater treatment system

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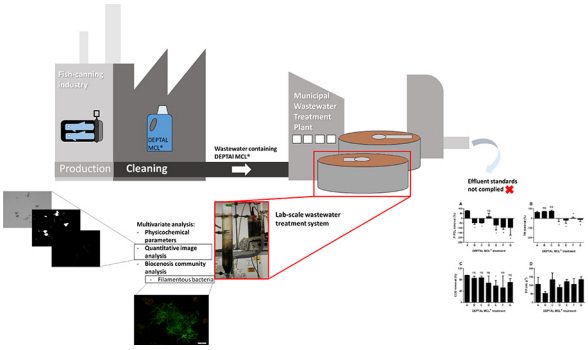
HIGHLIGHTS

- DEPTAL MCL® is a professional detergent and disinfectant used in agro-food industries.
- DEPTAL MCL® significantly hindered P-PO4 and TN removal.
- DEPTAL MCL® caused defloculation, in particular of intermediate size flocs.
- Multivariate analysis correlated filamentous bacteria abundance and affected parameters.
- Adequate pretreatment of wastewater containing DEPTAL MCL® is required.

GRAPHICAL ABSTRACT



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ABSTRACT

DEPTAL MCL® is a professional cleaning agent approved by the Portuguese Food Regulatory Authority and is used in agro-food industries, namely in fish canning industries in the north of Portugal. Its extensive use during cleaning procedures results in potential significant negative impacts on the performance of the downstream municipal wastewater treatment plant (WWTP). A lab-scale extended areation activated sludge wastewater treatment system, continuously fed by influent collected at a municipal WWTP, was used to assess the impact of a range of DEPTAL MCL® concentrations during 72 h. Despite distinct activated sludge community composition (due to its dynamic nature) and variations in real influent characteristics, a relevant impact was observed. DEPTAL MCL® effect was underscored through the use of a multivariate analysis using seventeen physicochemical operational factors and nineteen quantitative image analysis (QIA) parameters. DEPTAL MCL® exerted a severe negative impact on phosphorous (P-PO_4) removal, total nitrogen (TN) removal and sludge volume index (SVI). With increasing DEPTAL MCL® concentrations, both P-PO_4 and TN removal were affected and diminished proportionally. Moreover, several QIA parameters indicate defloculation when DEPTAL MCL® was present, in particular for intermediate size aggregates with significant impacts. Optical density of the effluent (Od_e), displayed an increase of effluent turbidity. Percentage of area covered by small aggregates (%$Area_{sml}$) was also significantly higher for the intermediate and higher DEPTAL MCL® concentrations tested. Principal component analysis exhibited 3 distinct ordenations: (i) control without addition of DEPTAL MCL®; (ii) addition of 0.03% and 0.06% and of (iii) 0.13 and 0.26% (v DEPTAL MCL®/v aeration tank). Canonical correspondence

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analysis (CCA) was used to correlate the physicochemical data, QIA and the filamentous bacteria species prevalence to DEPTAL MCL® concentration and incubation time. A time persistent DEPTAL MCL® effect was observed, underscoring the need of a pretreatment of wastewater containing this cleaning agent.

Abbreviations

General abbreviations

A	No DEPTAL MCL® was added
B	DEPTAL MCL® concentration 0.03% (v DEPTAL MCL®/v aeration tank)
C	DEPTAL MCL® concentration 0.06% (v DEPTAL MCL®/v aeration tank)
CCA	Canonical correspondence analysis
D	DEPTAL MCL® concentration 0.13% (v DEPTAL MCL®/v aeration tank)
E	DEPTAL MCL® concentration 0.13% (v DEPTAL MCL®/v aeration tank)
F	DEPTAL MCL® concentration 0.26% (v DEPTAL MCL®/v aeration tank)
G	DEPTAL MCL® concentration 0.26% (v DEPTAL MCL®/v aeration tank)
PCA	Principal component analysis
PERMANOVA	Permutational analysis of variance/multivariate analysis of variance
QIA	Quantitative image analysis
R	return line form clarifier
SC	Secondary clarifier
WS	Waste sludge
WWTP	Wastewater treatment plant

Physicochemical parameters

AF	Air flow
COD	Chemical oxygen demand
CODr %	Percentage chemical oxygen demand removal
F_{COD}/M	Food (expressed in chemical oxygen demand) to microorganism ratio
HRT	Hydraulic retention time
OD	Optical density
$P\text{-}PO_4$	Phosphorous
Q_i	Influent flowrate
Q_{ws}	Waste sludge flow rate
S_0	Influent chemical oxygen demand concentration
SRT	Solids retention time
SVI	Sludge volume index
TN	Total nitrogen
TSS	Total suspended solids
VSS	Volatile suspended solids
X	Biomass concentration

Quantitative image analysis parameters

TL/Vol	Total filaments length per volume
TL/TA	Total filaments length per total aggregates area
TA/Vol	Total aggregates area per volume
Deq	Equivalent diameterP – Aggregate perimeter
FF	Form factor
Conv	Convexity
Comp	Compactness
Round	Roundness
Eccen	Eccentricity
Robus	Robustness
LrgC	Largest concavity
RelArea	Area ratio
% Nb	Number percentage
% Area	Area percentage

Subscripts

AT	aeration tank
e	effluent
i	influent
int	intermediate aggregates
R	return line form clarifier
sml	small aggregates

1. Introduction

Worldwide fish consumption per capita is increasing approximately 1.5% each year, reaching nearly 20 kg per capita per year (FAO, 2018). In this regard, the total income produced by EU fish processing industry was estimated at EUR 32.5 Billion, according to Scientific, Technical and Economic Committee for Fisheries (STECF, 2019). For example *Canned tuna* is consistently among the most consumed fish species in Europe (EUMOFA, 2018). On the other hand, fish canning industries generate high water consumption due to thawing and washing processes which, consequently, produces large quantities of wastewater (approximately 18 m^3 $tonne^{-1}$ of commodities). This wastewater is renowned by its high load of contaminants such as nitrogen and organic compounds such as detergents, which require treatment prior to their disposal into the environment (Anh et al., 2021). Fish canning industries wastewater encompasses a highly variable chemical oxygen demand (COD), ranging between 1 and 50 g L^{-1}, and is mainly constituted by proteins and lipids (Chowdhury et al., 2010; Cristóvão et al., 2014). The use of detergent in the fish canning industries is due to regulations and directives on high standars of sanitary conditions in food processing plants. Nevertheless, the impact of detergents used in fish canning industries during mandatory regular cleaning procedures in the biological wastewater treatment has been coarsely disregarded. DEPTAL MCL® (HYPRED) is a professional grade alkaline chlorinated detergent and disinfectant, which is legally approved by the European Union to be applied in agro-food industrial facilities to meet food safety standards (European Parliament, 2002). Portugal is a major fish consumer country in the European Union reaching 60 kg per capita approximately (FAO, 2020). Furthermore, the use of DEPTAL MCL® is the main biocide agent used in fish canning industries to sanitize objects, equipment and furniture surfaces(fish canning industry communication). An important concern related with fish canning industries is that fishery facilities wastewater is solely subjected to a simple pretreatment, or none at all (Cristóvão et al., 2014; Cristóvão et al., 2016). The effective activity of chlorine alkaline DEPTAL MCL® against pathogenic microorganisms such as Listeria, *Staphylococcus* spp., *Escherichia coli* and other present on surfaces at a food processing plants, clearly denotes a broad spectrum of activity (Bagge-Ravn et al., 2003; Khamisse et al., 2012). Furthermore, the quantities required for the effective and systematic disinfection of a fishery facility, will comprehensively generate non-negligible amounts of this biocide. Thereofore, considering these two important factors, a problematic concentration of DEPTAL MCL®will reach the wastewater treatment plants (WWTP) and may considerably impact its performance. This issue, to the authors knowledge, was

not yet thoroughly evaluated. Antimicrobial action of chlorine cleaning agents is based on the oxidative interaction with sulfhydryl group of several enzymes, resulting in its denaturation. In addition, the microbial cell membrane is also disrupted due to chlorine high oxidizing reactivity (Bodík et al., 2008). A decrease of 60% of respiratory activity of activated sludge was reported when using a chlorine dose of 4.75 mg of Cl_2 per g of volatile suspended solids (VSS) within 20 min, or by applying 7.96 mg Cl_2 per g of VSS for 10 min (Caravelli et al., 2004). Despite extensive data available on the effect of chlorine on activated sludge, information about the potential impact of more complex detergents as DEPTAL MCL® in WWTP, particularly during the biological treatment, is still missing.

In this study, following an episode of extensive intoxication of the sludge biomass in a municipal WWTP in the North of Portugal, a lab-scale wastewater treatment system experiment was prefmormed to understand the potential effect of the biocide DEPTAL MCL® on this type of events. A wide-ranging concentration of DEPTAL MCL® was applied to the aeration tank (AT) of a lab-scale extended aeration wastewater treatment system. The system was operated in continuous, using sewage and activated sludge from a municipal WWTP from a region where no fish canning industries are present. Due to the high complexity and dynamic nature of activated sludge treatment, multivariate statistical methods were applied to provide an enhanced overview of the DEPTAL MCL® impact on the activated sludge wastewater treatment (Cristóvão et al., 2016; Legendre and Legendre, 2012). Moreover, these multivariate statistical methods were applied to comprehensively evaluate numerous parameters: physicochemical, floc structure profile (evaluated by quantitative image analysis (QIA) and microbiological abundance offilamentous bacteria.

2. Materials and methods

2.1. Lab-scale wastewater treatment system

The lab-scale wastewater treatment reactor comprised an aeration tank (AT) (2 L working volume) connected to a secondary clarifier (SC) (operating volume 1.9 L) (Fig. 1a). Therefore, the overall volume of the system was 3.9 L. At the bottom of the SC a return line form clarifier (R) ensured partial recycling of the activated sludge from the SC to the AT. The waste sludge (ws) was collected from R. Fig. 1 (b) exhibits a simplified scheme of the lab-scale system. The influent (i) used consisted in sewage collected immediately after the preliminary treatment of a municipal WWTP (Braga, Portugal; coordinates: 41°33′59.6"N 8°26′44.4"W). The AT activated sludge was collected from the same municipal WWTP. The hydraulic retention time (HRT) was approximately 28 h. Excess sludge was discarded from the R at a rate of 1.25 mL h^{-1}. The dissolved oxygen value in the AT was maintained between 1 and 3 mg L^{-1} by using a precision gas mass flow controller located downstream to an air pump.

2.2. DEPTAL MCL®

To evaluate the disinfectant and detergent DEPTAL MCL® comprehensive impact on activated sludge based secondary treatment, concentrations at 0.03; 0.06; 0.13; 0.26% (v(DEPTAL MCL®)/v(AT) and a control without addition DEPTAL MCL® were tested. Each concentration of DEPTAL MCL® was injected on the AT at the beginning of the experiment. The authors assumed that in control condition no DEPTAL MCL® is present in the influent. This was due to the absence of fish canning industries or major food processing facilities in the vicinity of the WWTP where it was collected. The concentrations 0.13% and 0.26% were analyzed in two independent assays, to verify the reproducibility of the results. For simplification purposes DEPTAL MCL® concentrations are represented by the following acronyms: no addition – A; 0.03% – B; 0.06% – C; 0.13% – D and E; and 0.26% – F and G. The highest tested concentration is approximately 8-fold higher than the lowest, to cover a broad range of concentrations. The reactor was re-inoculated and stabilized for at least 15 days before each experiment.

2.3. Physicochemical parameters

The physicochemical parameters were collected 24 h, 48 h and 72 h after the injection of DEPTAL MCL® and analyzed according to standard methods (APHA, 1995). System temperature was 21.6 ± 1.5 °C.

Physicochemical parameters used to calculate the system operational parameters included: biomass concentration in the influent (TSS_i and

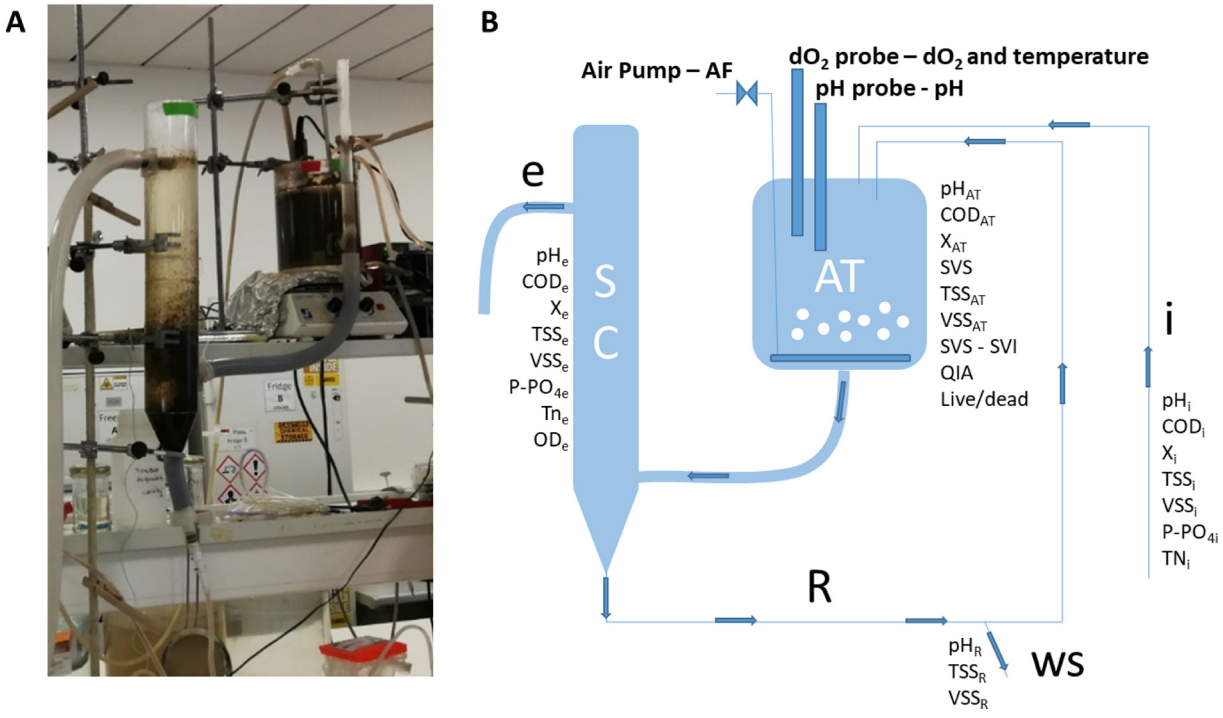

Fig. 1. A - Lab-scale wastewater treatment system. B - Schematic representation of the lab-scale wastewater treatment system and collected parameters.

VSS_i), aeration tank (TSS_{AT} and VSS_{AT}), and in the effluent (TSS_e and VSS_e). Settleability determined by settled volume of sludge was used to estimate the sludge volume index (SVI) (Metcalf and Eddy, 2003).

Soluble COD concentration was determined by COD cuvette test LCK314 and LCK514 (Hach), to estimate percentage of COD removal efficiency. The solids retention time (SRT) and food (expressed in COD) to microorganism ratio (F_{COD}/M) were determined according to Metcalf and Eddy (Metcalf and Eddy, 2003).

The soluble phosphorous (P-PO_4) and total nitrogen (TN) concentrations were estimated using LCK 350 (range of 2.0–20.0 mg L^{-1}) and LCK 338 (range of 20–100 mg L^{-1}) cuvette test (HACH, Manchester, GB), respectively and applied according to the manufacturing protocol. These tests present a Confidence interval of 95%. LCK 350 tests are in conformity with ISO 8466-1 and DIN 38402 A51 and the LCK 338 are in conformity with ISO 8466-2 and DIN 38402 A51. Phosphorous (P-PO_4) removal and total nitrogen (TN) removal were also determined. Additional detailed information is available in supplementary data.

2.4. Quantitative image analysis (QIA)

2.4.1. Image processing and analysis in bright-field images

Activated sludge morphology was assessed by quantitative image analysis (QIA) at each time point: 24 h, 48 h and 72 h after the injection of DEPTAL MCL®. Three slides per sample, and for each slide a sample volume of 10 μL was covered with a 20 mm × 20 mm cover slip for visualization and image acquisition. Images were acquired in the upper, middle, and bottom of the slide resulting in a total of 150 images (3 × 50 images $slide^{-1}$), with more than 8000 aggregates per sample in average. The slides were analyzed using a Olympus BX51 optical microscope (Olympus, Japan), at 100 × total magnification, coupled with an Olympus DP72 camera (Olympus, Japan). Images were acquired at 1360 × 1024 pixels and 8-bit format through the commercial software Cell^B (Olympus). The image processing and analysis procedure was based on the recognition of aggregates and filamentous bacteria in grayscale images using a specifically developed program in Matlab 7.5 (The Mathworks, Natick, MA). A more detailed description of the image processing methodology can be found in the work reported by Mesquita and authors (Mesquita et al., 2009). A cross-correlation analysis between all the collected data from QIA was then performed to reduce the dataset, leading to the exclusion of one variable for each pair presenting a correlation factor above 90%. The definition of each QIA parameter used in this work can be found in supplementary material. Fig. S1 shows an illustrative bright-field image with corresponding aggregates and filaments binary images obtained from the QIA procedure. Data comprising physicochemical and QIA parameters may be observed in Table S2.

2.5. Biological parameters

An Olympus CX41 microscope was used to observe stained smears (Gram or Neisser colored) of mixed liquor from AT, to identify filamentous bacteria based on their morphological characteristics (dos Santos et al., 2015).

2.6. Viability assay

At each time point, 1 mL of mixed-liquor was collected from AT for microbial viability evaluation (live/dead assay) based upon membrane integrity to differentiate live from dead populations (Boulos et al., 1999). Each sample was stained using a combination of two nucleic acid stains: green fluorescent SYTO™ BC (Invitrogen, USA) for live cell staining and red fluorescent propidium iodide (Sigma, Germany) for dead cell staining, at a final concentration of 0.2 μM and 3 μM, respectively. After incubation in the dark for 10 min at room temperature, 10 μL of stained suspension was spotted onto a microscope slide with a 20 mm × 20 mm cover slip for visualization and image acquisition with an epifluorescence microscope BX51 (Olympus, Japan) using FITC (EX 470 – EM BP490/520 nm) and TRITC (EX 530 – EM BP550/590 nm) filters to visualize live (green)/damaged (yellow) and dead (red) microorganisms, respectively. Multiple images (more than 10 for each sample) were captured with a DP72 digital camera (Olympus, Japan).

2.7. Statistical analysis

Activated sludge secondary treatment comprises a complex and dynamic set of parameters which is comprehensively analyzed using multidimensional diagrams (Legendre and Legendre, 2012). - D'Agostino & Pearson omnibus normality test was used to test the Gaussian distribution of P-PO_4 removal, TN removal, COD removal, SVI, equivalent diameter of intermediate aggregates (Deq_{int}) percentage area of small aggregates (%$Area_{sml}$), and effluent optical density (Od_e). COD removal did not pass the test, thus it was analyzed using Kruskal-Wallis with a Dunn's multiple comparisons, a non-parametric test. A Brown-Forsythe test ensured the variance homogeneity of P-PO_4 removal, TN removal, and SVI. Then, a One-way ANOVA and a Post hoc test Dunnett's multiple comparison was used to test if the DEPTAL had any effect on P-PO_4, TN removal and SVI. These analysis were performed using Graphpad Prism 6 (version, 6.01).

QIA parameters used were preselected after a cross correlation analysis. Physicochemical and QIA data were normalized and a principal component analysis (PCA) was established. Table S1 comprises the PCA analyzed parameters. An Euclidean distance resemblance matrix was applied and a permutational analysis of variance/multivariate analysis of variance (PERMANOVA) to assess the significance of factors: time, DEPTAL MCL® concentration and their interaction. A PERMANOVA was also applied to the biological data (*P-perm* < 0.05). A canonical correspondence analysis (CCA) was used to perform an overall correlation among: physicochemical data, QIA results and filamentous bacteria abundance.

PCA and PERMANOVA analysis were performed using Primer 6 (version 6.1.16) & PERMANOVA + (version 1.0.6, PRIMER-E) software. CCA were obtained using Paleontological Statistics (PAST version 3.23) software (Hammer et al., 2001). The presented plots were generated using GraphPad Prism (version 6.01, GraphPad Software Inc.).

3. Results and discussion

The P-PO_4 removal, TN removal, COD removal and SVI are important parameter to assess the performance of a WWTP. When present, DEPTAL MCL® clearly impacts these parameters. P-PO_4 removal was significantly affected by exposure to increasing concentrations of DEPTAL MCL® (ANOVA, $p = 0.0007$; Fig. 2A). The highest P-PO_4 removal (78%) was found in the control treatment (A) where no DEPTAL MCL® was added. All DEPTAL MCL® concentrations promoted a decrease in P-PO_4 removal (Dunnett's test, $p < 0.05$), with the highest reduction in treatment G (−95%). A negative relationship was found between P-PO_4 removal and increasing DEPTAL concentrations (linear regression, $p = 0.0025$, $r^2 = 0.3885$, $Y = -427.7\times + 16.76$).

Regarding TN removal, it was also significantly affected by exposure to DEPTAL MCL® (ANOVA, $p < 0.0001$; Fig. 2B). DEPTAL MCL® concentrations from D to G promoted a decrease in N removal (3 to −18%) compared with control treatment A (63%) (Dunnett's test, $p < 0.05$). A negative relationship was found between N removal and increasing DEPTAL concentrations (linear regression, $p = 0.0002$, $r^2 = 0.5273$, $Y = -315.6\times + 65.28$).

COD removal was also significantly affected by exposure to increasing concentrations of DEPTAL MCL® (Kruskal-Wallis, $p = 0.0457$; Fig. 2C). The higher COD removal (96%) was found in the control treatment (A). Only concentration E (59%) was significantly different from control (Dunn's test, $p < 0.05$). Similarly to P-PO_4 and TN removal, a negative relationship was found between COD removal and increasing DEPTAL concentrations (linear regression, $p = 0.0112$, $r^2 = 0.2935$, $Y = -126.9 + 90.01$). Finally, SVI was also significantly affected by exposure to DEPTAL (ANOVA, $p = 0.0273$; Fig. 2D). The lowest SVI was found in treatment B (53 mL) and the highest in treatment G (135 mL). No significant differences

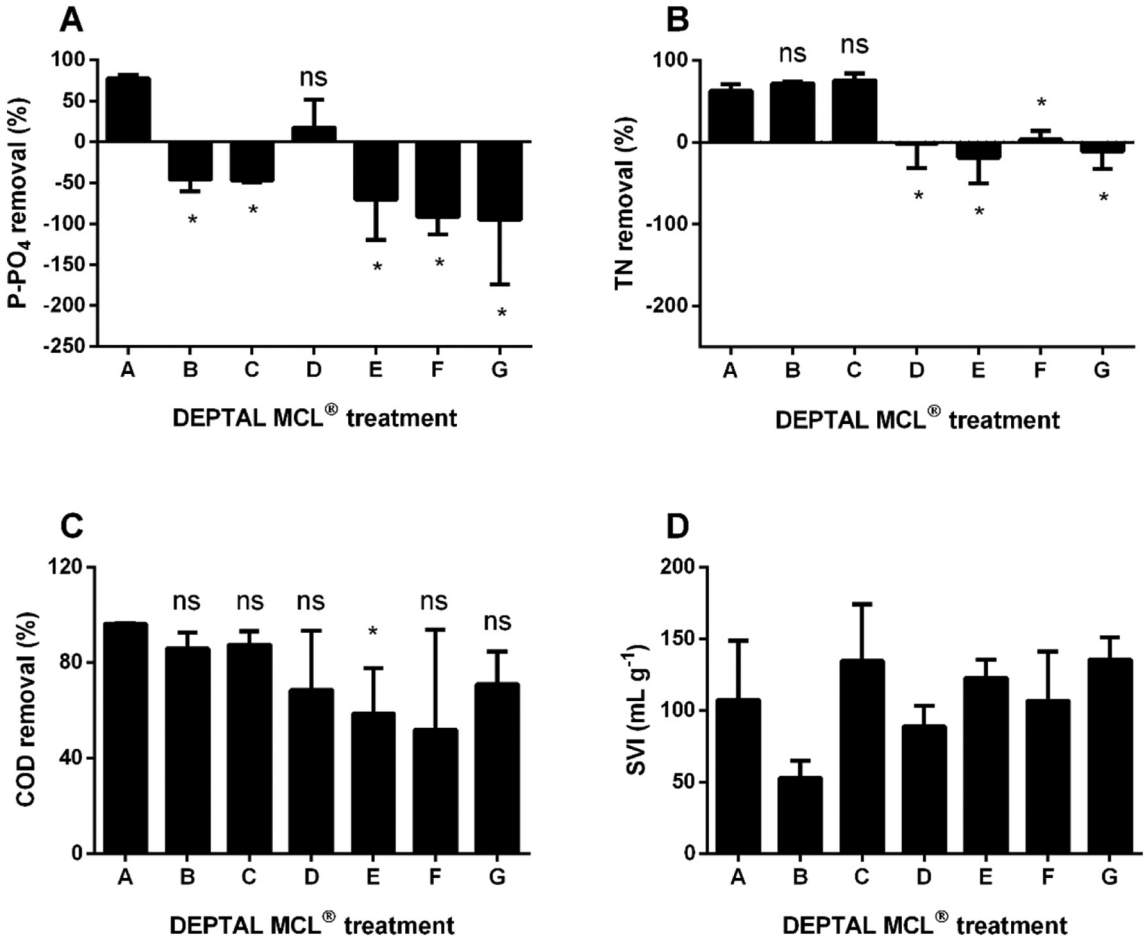

Fig. 2. Effects of increasing concentrations of DEPTAL MCL® on: A – percentage of P-PO_4 removal, B - percentage of TN removal, C – percentage of COD removal and D – SVI. Values are mean of all exposure times (24, 48 and 72 h). DEPTAL MCL® concentrations: A – no addition, B - 0.03%, C - 0.06%, D and E - 0.13%, F and G - 0.26% (v(DEPTAL MCL®)/v(AT). Differences were tested against treatment A (control) by a Dunnett's multiple comparisons test (P-PO_4 removal, TN removal, SVI) or Dunn's multiple comparisons test (COD removal); $^*p < 0.05$; ns, non significant differences. Mean (+SD, $n = 3$).

were observed between control treatment (A) and others treatments (Dunnett's test, $p > 0.05$). Also, no relationship was found between SVI and increasing DEPTAL concentrations (linear regression, $p > 0.05$).

Dispite no SVI correlation was observed, there are some evidences that highlight the occcurance of defloculation, such as OD_e, $\%Area_{sml}$, Deq_{int} and the drastic loss of $Comp_{int}$ (Fig. 3).

The clear reduction of $Comp_{int}$ and Deq_{int} may be correlated with defloculation of these aggregates (Fig. 3A and B). Which results in a significant higher $\%Area_{sml}$ as observed in Fig. 3C. Finally, an increase of the turbidity of the effluent may be due to the inefficient sedimentation of the flocs, also due to the defloculation. Furthermore, apparently the activated sludge was able to mitigate the alkaline nature of DEPTAL MCL®, since no clear differences were observed (Fig. S3A).

Fig. 4 displays the PCA for physicochemical and QIA parameters of the aggregates, comprising more than half of the percentage of distribution and Table S1 comprises the eigenvectors.

PCA is a reader friendly approach to simplify the complexity of extensive data. PCA highlights the trends of data distribution in geometrically projected principal components (PC). The PC1 and PC2 must be uncorrelated and their percentage provides information regarding the coverage of variance (Lever et al., 2017).

DEPTAL MCL® concentrations B to G present a similar distribution within the PCA X-axis, whereas the control (A) has a completely distinct ordination distribution. Lower DEPTAL MCL® concentrations B and C present a ordination with a distinct PCA Y-axis spatial distribution from the ordination encompassing intermediate (D and E) and highest concentrations (F and G).

Control (A) important eigenvectors comprise: $Comp_{int}$, largest concavity of small aggregates ($LrgC_{sml}$) and form factor of intermediate aggregates (FF_{int}). Lowest concentration (B) relevant eigenvectors are compactness of small aggregates ($Comp_{sml}$), convexity of intermediate aggregates ($Conv_{int}$) and roundness of intermediate aggregates ($Round_{int}$). In both control (A) and lowest concentration (B) the eigenvectors denote the importance of intermediate aggregates morphology, indicating that lowest DEPTAL MCL® concentrations displays an mild impact on the intermediate aggregate structure. Low concentration (C) important eigenvectors are the total suspended solids at AT (TSS_{AT}), volatile suspended solids at AT (VSS_{AT}) and air flow (AF), exhibiting clear effect at AT. AF needed to maintain the dissolved oxygen within the established range is directly correlated to activated sludge activity (Gallé et al., 2019). These parameters were higher then all other tested concentations (including the control), there is not a clear correlation with the influent solids or any other parameter (Fig. S3B, S3C and S3D). Relevant eigenvectors which lead to the PCA ordinations for the intermediate (D and E) and higher (F and G) concentrations of DEPTAL MCL® include number percentage of small aggregates ($\%Nb_{sml}$), area percentage of small aggregates ($\%Area_{sml}$) and effluent optical density (OD_e). This clear ordination distinction mainly based on the size of small aggregates and OD_e denotes a deflocculation event, due to the changes on the aggregates number and size, in addition to lower settleability at the SC (Andreadakis, 1993). This ordination clearly demonstrates the high impact of intermediate (D and E) and high (F and G) DEPTAL MCL® concentrations on the biomass size, structure and the loss of key wastewater treatment performance parameter: settleability.

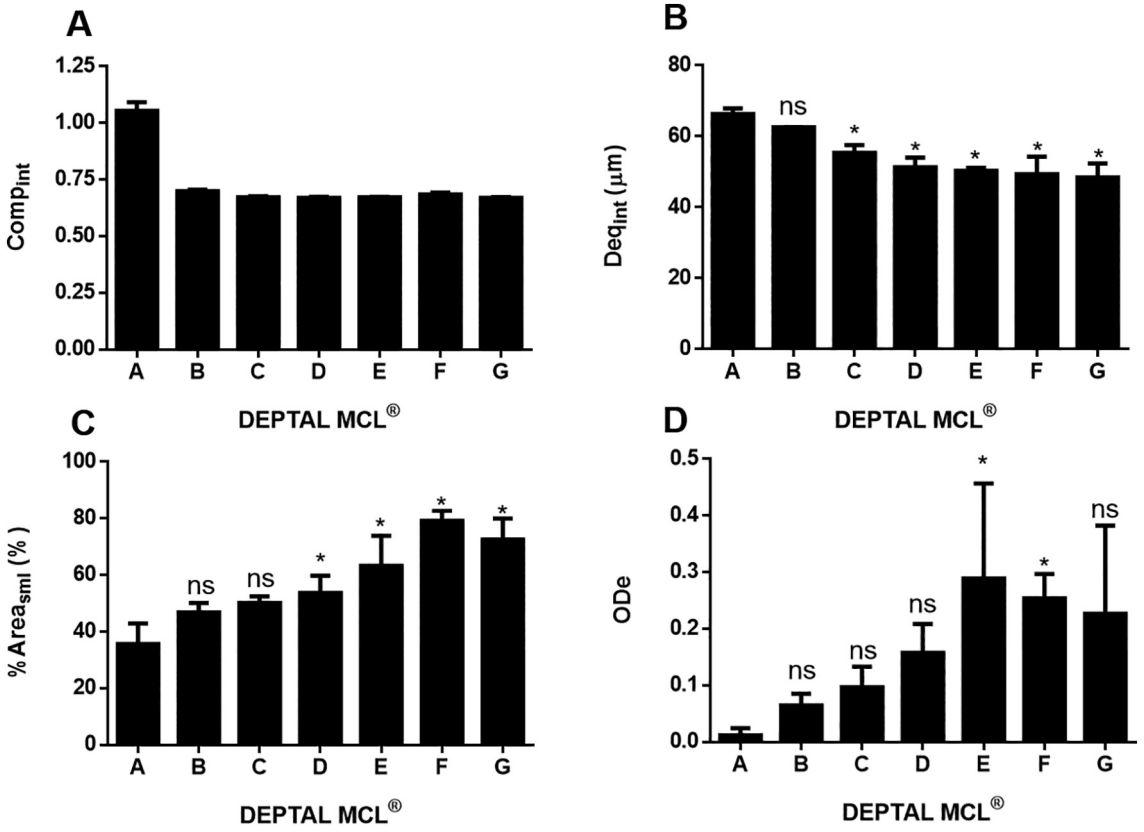

Fig. 3. QIA and physicochemical parameters: A – $Comp_{int}$, B – Deq_{int}, C - $\%Area_{sml}$ and D – OD_e. DEPTAL MCL® concentrations: A – no addition, B - 0.03%, C - 0.06%, D and E - 0.13%, F and G - 0.26% (v(DEPTAL MCL®)/v(AT). Differences were tested against treatment A (control) by a Dunnett's multiple comparisons test; $*p < 0.05$; ns, non significant differences. Mean (+SD, n = 3).

The PCA results are corroborated by the PERMANOVA analysis which indicate DEPTAL MCL® as a significant factor (*P*-perm = 0.007), highlighting the effective impact of DEPTAL MCL® on the physicochemical parameters and floc structure of the activated sludge. Time presented a non-significant value (*P*-perm = 0.08) which denotes the persistent DEPTAL MCL® effect within the analyzed incubation period (72 h), albeit

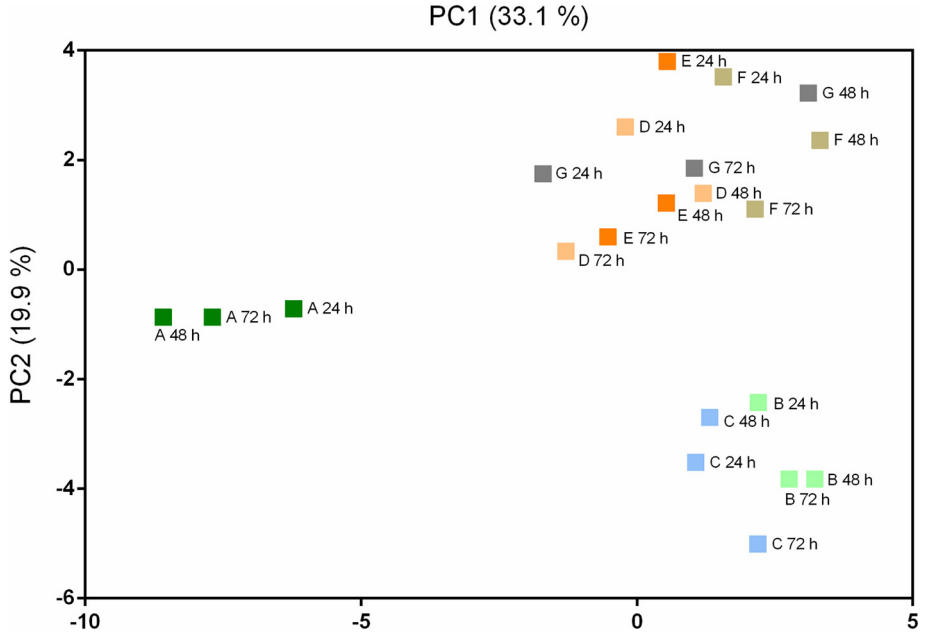

Fig. 4. Principal component analysis (PCA) comprising the physicochemical and quantitative image analysis (QIA) data after 24 h, 48 h and 72 h of DEPTAL MCL® exposure. DEPTAL MCL® concentrations: A – no addition, B - 0.03%, C - 0.06%, D and E - 0.13%, F and G - 0.26% (v(DEPTAL MCL®)/v(AT).

the 28 h of HRT. This may be correlated with a possible interaction and perseverance of DEPTAL MCL® with the solids, since the lowest recorded SRT was nearly 3.6 days (Table S2).

Regarding the biological communities, DEPTAL MCL® concentration was the sole significant parameter forfilamentous bacteria (*P*-perm = 0.027). Fig. 5 depicts the average prevalence of the filamentous bacteria. It is clear that when no DEPTAL MCL® was added a higher biodiversity of filamentous bacteria was observed (77.8% of bacteria identified). Type 0041/0675 did not display considerable differences among all tested conditions. Type 0092 prevalence was lower in all tested concentrations of DEPTAL MCL®. Type 021 N prevalence was lower in B, C and D, whereas considerably higher in F and G. *Thiothrix* spp. and *Gordonia* spp. were only observed in A. *Microthix parvicella* average prevalence was lower in B and C and considerably higher in G. *Nostocoida limicola* was identified in A and E. *Haliscomenobacter hydrossis* and *Sphaerotilus natans* were not observed in A. *H. hydrossis* was only identified in F and G, the higher concentrations of DEPTAL MCL®. *S. natans*, observed solely in D and E depicted a considerably higher average prevalence in condition E.

CCA displays the overall correlation between physicochemical, QIA and biological data. Wastewater treatment processes performance and filamentous bacteria profile and dynamics had been widely correlated (dos Santos et al., 2015). In Fig. 6 it is observable the distribution of the different concentrations of DEPTAL MCL® at different time points, as well as the associated filamentous bacteria species, the physicochemical and QIA eigenvectors. CCA divides the filamentous bacteria species within five different ordinations.

The CCA ordination of the filamentous bacteria communities according to physicochemical parameters and QIA data showed that the first axis explained 44.38% of the total variance in filamentous bacterial communities and separated the control communities from all the remaining. The second axis explained 26.58% of the total variance and distinguished communities from intermediate and higher DEPTAL MCl® concentration.

Filamentous bacteria *Gordonia* spp., *Thiothrix* spp. and *Nostocoida limicola* are associated with control condition (A). Interestingly, *N. limicola* is known as chlorine resistant Actibacteria, thus its prevalence was expected to be associated to higher DEPTAL MCL® concentrations, which was not the case (Séka et al., 2001). The Actinobacteria *Gordonia* spp. is widely recognized as foaming agent in activated sludge (Guo et al., 2015), however, no relevant foam was observable in the reactor, independently of the DEPTAL MCL® concentration tested. Therefore, *Gordonia* spp. relative abundance in control (A) may be correlated with its ubiquitous presence in activated sludge. The Proteobacteria *Thiothrix* spp. and Actinobacteria *N. limicola* are known to cause bulking events, however no bulking events were observed (Jenkins et al., 2004). *Gordonia* spp., *Thiothrix* spp. and *N. limicola* relative abundance was associated to eigenvectors of aggregates morphology, in particular of intermediate aggregates, as previously discussed. Which denotes a correlation between good depuration conditions (A - control) and intermediate aggregates morphology. *Sphaerotilus natans* relative abundance was correlated with intermediate DEPTAL MCL® E and D (24 h) concentration. *S. natans* belongs to Betaproteobacteria and is the most extensively studied bulking events in activated sludge systems worldwide (Ferreira et al., 2021). Furthermore, this bacterium is commonly found in municipal WWTP. A ordination comprising DEPTAL MC® concentrations B and C 24 h D 72 h and F 72 h observable. The filamentous bacteria with a distribution nearer to this ordination include Type 0092, Type 0041/0675 and *Microthrix parvicella*. *M. parvicella*, referred as one of the most ubiquitous filamentous bacteria in the activated sludge, is chlorine resistant and responsible for bulking and foaming events (Séka et al., 2001; Rossetti et al., 2005). In addition, *M. parvicella* is associated to SRT eigenvector, an operational parameter usually correlated with its prevalence (Rossetti et al., 2005). More recently, *M. parvicella* overgrowth was associated to low operational temperatures (15.30 ± 0.26 °C) and influent COD constantly below 100 g COD kg^{-1} mixed liquor suspended solids day^{-1} (Fan et al., 2017). Average influent COD for all experiments was approximately 3 mg COD kg^{-1} VSS_{AT} day^{-1} and *M. parvicella* bulking was not observed within the experimental temperature (1.4-fold higher). This may denote that temperature is a more determinat factor for *M. parvicella* bulking events than low inffluent COD, for this system. Type 0092, Type 0041/0675 are referred as bacteria able to thrive in activated sludge with high concentrations of recalcitrant nutrients. Relevant eigenvectors for these bacteria include $\%Area_{sml}$ and $\%Numb_{sml}$ undercoring aggregates defloculation. Type 21 N can be ascribed to C 48 and 72 h, D 48 h and F 72 h. Different species of Type 021 N demonstrated distinct susceptibility to chlorine, withstanding between 0.015 mg to 80 mg of Cl_2 g^{-1} of suspended solids (Séka et al., 2001; Guo et al., 2012). This may explain its prevalence in the intermediate and higher concentrations (C, D and F). The presence of *Haliscomenobacter hydrossis* is in line with the highest DEPTAL MCL® concentrations (F 24 h, G 24 and 48 h). *H. hydrossis* prevalence and overgrowth has been associated with dissolved oxygen depletion, nevertheless, in this case, it was not negatively correlated with the AF eigenvector (Jiao et al., 2018).

Fig. 7 highlights the defloculation by depicting the presence of smaller aggregates with the increasing concentration of DEPTAL MCL®. Some loss of viability of the bacteria present is also observable.

In summary, for this wastewater treatment system, *Gordonia* spp., *Thiothrix* spp. and *N. limicola* were prevalent in control assay. *S. natans*, Type 0092, Type 0041/0675, *M. parvicella* and Type 021 N were associated to low and intermediate DEPTAL MCL® concentrations and *H. hydrossis* were associated to the highest degree of loss of system performance (higher DEPTAL MCL® concentrations). All DEPTAL MCL® concentrations exhibited defloculation, which severely affects the effluent final quality and adequacy, compromising the effluent legal requirements.

4. Conclusions

DEPTAL MCL® is a chlorine alkaline detergent, thus its biocidal action on the activated sludge biocenosis may be direct or indirect if this biocide exerts an unfavorably modification of the environmental parameters.

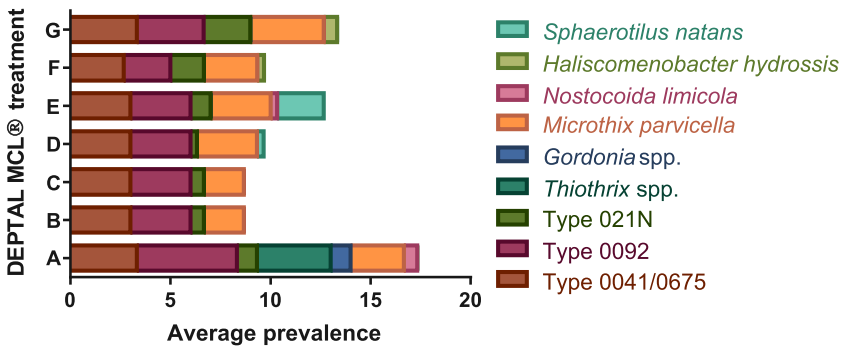

Fig. 5. Average prevalence of filamentous bacteria. DEPTAL MCL® concentrations: A – no addition, B - 0.03%, C - 0.06%, D and E - 0.13%, F and G - 0.26% (v(DEPTAL MCL®)/v(AT).

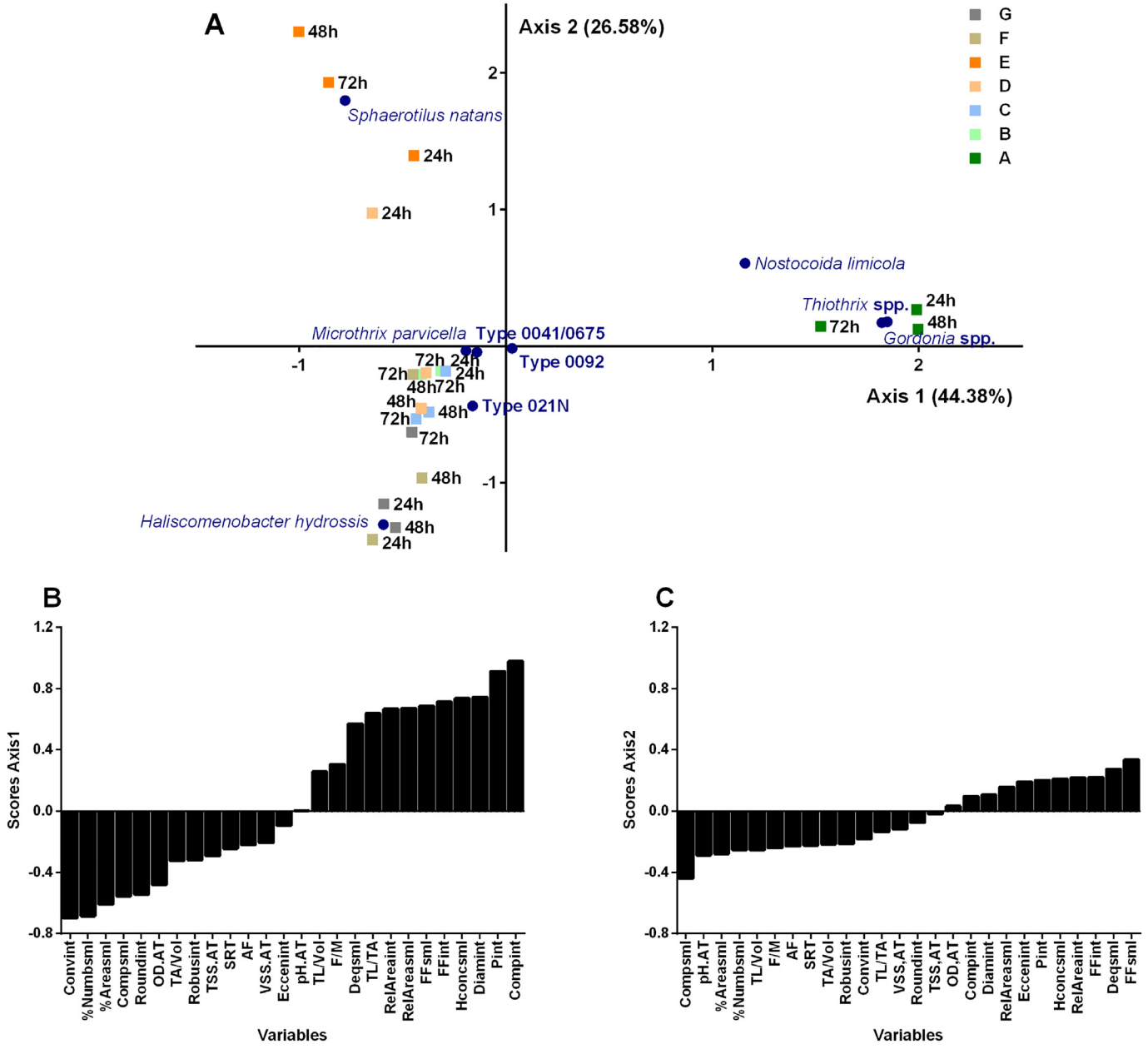

Fig. 6. A - CCA displaying filamentous bacteria prevalence, using physicochemical parameters and QIA data. DEPTAL MCL® concentrations: A – no addition, B - 0.03%, C - 0.06%, D and E - 0.13%, F and G - 0.26% (v(DEPTAL MCL®)/v(AT). B and C – tested variables score for Axis 1 and 2, respectively.

Using multivariate analysis comprising the physicochemical parameters, the QIA data and the biological data of filamentous bacteria of the activated sludge, it was possible to observe a clear negative impact of DEPTAL MCL® on activated sludge wastewater treatment system. This negative impact was independent of the inevitable variations on the activated sludge biocenosis community and unpredictability of real influent constitution. It must be mentioned that an individual assessment of each parameter exhibited a traceable impact of DEPTAL MCL® in the wastewater system. However, some of classical parameter for evaluating aggregates settleability such as SVI, failed to denote defloculation. Nevertheless, the holistic approach using multivariate analysis, allowed to clearly seperate the concentration by ordenations, providing a bigger picture encompassing important biological activated sludge communities such as filamentous bacteria. The findings of this work largely corroborate the operational issues experienced at a municipal WWTP which received influent containing DEPTAL MCL®. Available comprehensive information on filamentous bacteria is scarce and, to the authors knowledge, absent in multivariate studies. This approach should be further implemented in the highly complex, dynamic, and multifactorial process that is wastewater treatment, to establish solid biologic correlations, since filamentous bacteria play an important role in activated sludge process. Low levels of these microorganisms are required for adequate flocculation and to avoid the formation of viscous foam can lead to several operational problems (Jenkins et al., 2004; Mielczarek et al., 2012). Finally, all wastewater producing industries should not only focus on the control and evaluation of their regular wastewater. In this regard, a special concern must be warranted, for instance, during cleaning procedures. If the mitigation of levels of detergents or disinfectants during cleaning procedures are not feasible due to food safety reasons, the wastewater from these events must be analyzed and submitted to an adequate pretreatment. This will ensure a non problematic range of concentration of this products for the downstream activated-sludge wastewater treatment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.155957.

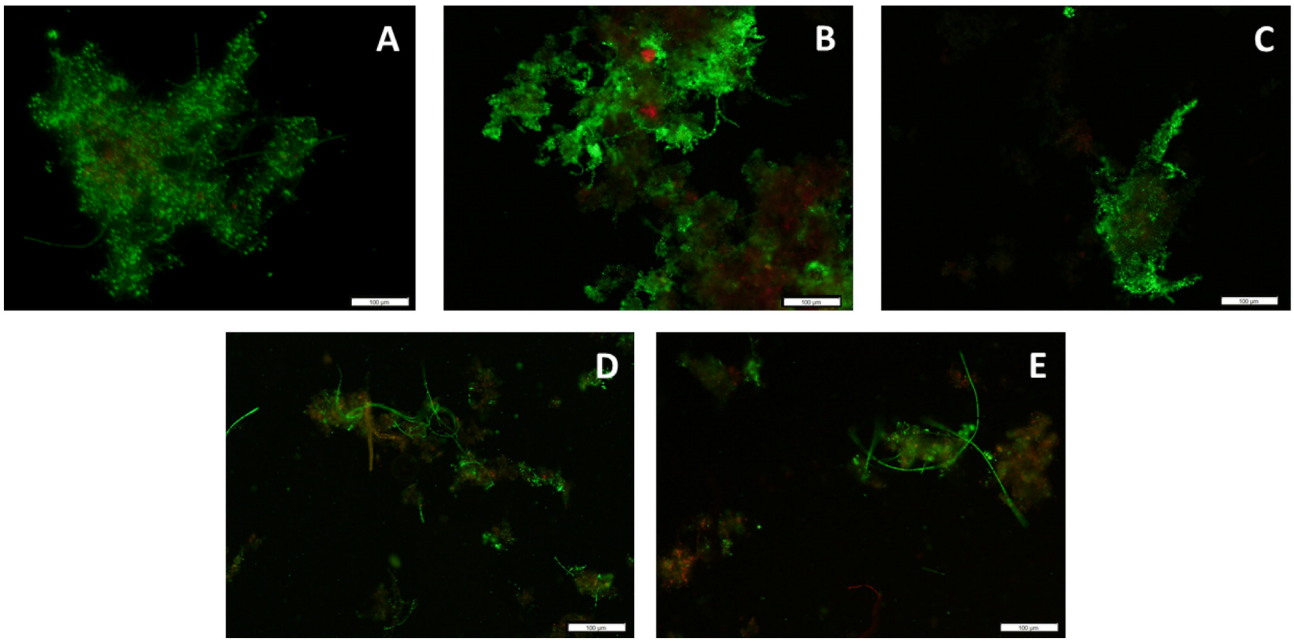

Fig. 7. Multi-channel epifluorescence microphotograph of A – condition A at 48 h; B - condition B at 48 h; C - condition C at 48 h; D - condition D at 48 h; E - condition F at 48 h.

CRediT authorship contribution statement

Jorge Padrão: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Vânia Ferreira:** Investigation, Methodology, Writing – original draft. **Daniela P. Mesquita:** Conceptualization, Data curation, Methodology, Project administration, Software, Validation, Writing – original draft. **Susana Cortez:** Investigation, Methodology, Writing – original draft. **Nicolina Dias:** Investigation, Methodology, Writing – original draft. **M. Salomé Duarte:** Investigation, Methodology, Writing – original draft. **Gonzalo Tortella:** Data curation, Formal analysis, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Isabel Fernandes:** Data curation, Formal analysis, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Manuel Mota:** Funding acquisition, Supervision, Validation, Writing – review & editing. **Ana Nicolau:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

All authors declare that they do not possess any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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