

Title

1
2 **Insights into the optimisation of the Clean-In-Place technique: cleaning,**
3
4 **disinfection, and reduced environmental impact using ozone-based formulations**
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Keywords

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43 Ozone, Starch, Cleaning-In-Place, Toxicity, Disinfection
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Abstract

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51 **Chemical and** food industries are focusing on **reducing** the economic and environmental
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53 impact of cleaning operations. This study **aims to analyse the** use **of** alkaline ozonated
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55 formulations to remove starch adhering to stainless steel surfaces, improving disinfection
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57 of mature biofilms with *Listeria* and *Pseudomonas*, and reducing the environmental
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1 impact of wastewater. Three key cleaning parameters have been analysed in a lab-
2 simulated Clean-in-Place system (CIP): temperature (20-60°C), time (20-120 min), and
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4 cleaning formulations containing ozone and surfactant (linear alkylbenzene sulfonate,
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6 alkylpolyglucoside, fatty ethoxylated alcohol, lauramine oxide) were assayed. Higher
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8 temperature, time, and ozone concentration improved starch removal and disinfection of
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10 the stainless-steel surfaces, as well as the environmental impact of cleaning wastewater.
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12 Therefore, ozone-based CIP protocols could provide new opportunities to achieve
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14 cleaner, greener, and safer industries by intensifying cleaning, disinfection, and
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16 wastewater treatment in one step, saving operational costs compared to conventional CIP
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18 techniques.
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26 **1. Introduction.**

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28 Modern industries require high levels of hygiene to safeguard product quality, where
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30 Clean-in-Place systems (CIP) are widely implemented as they provide fast, consistent,
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32 and reproducible high-quality cleaning (Moerman et al., 2014). CIP protocols combine
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34 rinsing, cleaning and sanitising fluids to eliminate fouling, microorganisms, and
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36 pathogens from surfaces (Wilson, 2018), depending on the properties of the deposits
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38 (Fryer and Asteriadou, 2009). However, those cleaning operations have a significant
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40 impact on the total operational costs of the processing plant, of which cleaning cost could
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42 be broken down to 44% water, 30% energy and 26% chemicals (Timmerman, 2014).
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47 Starch is a versatile additive commonly used in food, paper, chemical and pharmaceutical
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49 industries (Waterschoot et al., 2015)). The formation of starch deposits during product
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51 processing is frequent, especially under thermal treatment, where fouling is the major
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53 drawback (Liu et al., 2006). Amylaceous soils could be considered as water-insoluble soil
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55 with greater removal problems according to the classification made by Basso et al.(2017).
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1 Starches can generate hard-to-remove deposits, especially in thermal processes (Liu et
2 al., 2006), requiring the use of alkaline chemicals and high temperatures (Herrera-
3 Márquez et al., 2019). The complexity of the cleaning may further increase as the deposit
4 composition changes (Herrera-Márquez et al., 2020). Moreover, cleaning devices are
5 often oversized and designed according to worst-case scenarios (Murcek et al., 2021),
6 making therefore necessary the optimization of these systems as well as of the cleaning
7 parameters in order to reduce the consumption of energy, water, time and chemicals.
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17 Minimizing the environmental impact of industrial processes also requires the efficient
18 use of water and energy, reducing the waste formation. The highest operational
19 expenditure is associated with water consumption; e.g. cleaning requires 1/3 of the water
20 consumed during beer production (Fillaudeau et al., 2006). Currently, wastewater taxes
21 paid by the industries are directly related to effluent pollution, containing carbohydrates,
22 fats, proteins, or cleaning products (Guzel-Seydim et al., 2004). Therefore, going green –
23 modifying current cleaning/disinfection formulations using chemicals with lower toxicity
24 and higher biodegradability – could reduce the process costs.
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Aqueous and gaseous ozone treatments are effective against microbial contaminants
(Martinelli et al., 2017) allowing the possibility to reuse reconditioned water (Gómez-
López et al., 2015). Compared with chlorine, ozone needs a shorter contact time and lower
concentration in order to exert its disinfectant effect (Rosenblum et al., 2012) being a
powerful oxidant for water treatment (Ngwenya et al., 2013). Ozone is considered as
GRAS (Generally Recognized as Safe) technology by the FDA (United States Food and
Drug Administration), and BAT (Best Available Technique) by the European Union
(Industrial Emissions Directive 2010/75/EU, 2019). The use of ozone presents some
limitations and problems. For instance, N-nitrosodimethylamine (Kimura and Ortega-
Hernandez, 2019) and hydroxylamine (Heeb et al., 2017) are disinfection byproducts

1 formed during water disinfection with ozone under certain conditions (ozone dosages,
2 pH) and water components (humic acids or sulphate, bicarbonate and bromide ions).
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4 Sleeper and Henry (2002) also concluded that the use of ozone should be limited in their
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6 applications with elastomers, protective coatings, joint sealants, or metals, although glass,
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8 HDPE, or stainless steel showed not to be affected by the use of ozone. However, ozone
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10 disinfection treatment is better than hypochlorite treatments from corrosion, economic,
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12 and environmental perspectives (Romanowski et al., 2020) as it reduces the pollutants
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14 formation in effluents (Ghughe and Saroha, 2018) improving the cleaning and disinfection
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16 in the food industry, where acts as a bactericidal and virucidal agent (Pandiselvam et al.,
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18 2019).

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24 Ozonation has been incorporated into the food industries to modify the properties of
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26 starch, the sanitation of equipment (Masotti et al., 2019), and the treatment of wastewater
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28 because it offers several advantages:

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31 • Ozonation affects the rheological properties of food materials, modifying the
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33 properties of pasting, clarity, texture, swelling, and solubility of starches (Castanha et
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35 al., 2017, 2019b). However, the prevalence of ozone is minimal compared with other
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37 technologies, being necessary the standardization of the ozone processes to better
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39 understand the effect of different factors (Sivaranjani et al., 2021).
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43 • Ozone can remove fats (Jurado-Alameda et al., 2012a), proteins (Jurado-Alameda et
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45 al., 2014), and starches (Avila-Sierra et al., 2020) in cleaning processes.
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49 • Ozone is a strong oxidant with a wide antimicrobial spectrum (Pandiselvam et al.,
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51 2019). Ozone could be a disinfectant in CIP devices (Castanha et al., 2017), avoiding
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53 additional rinsing stages and/or the presence of undesired pollutants in the
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55 wastewater, e.g. pollutants formed from the decomposition of chlorine (Brodowska
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57 et al., 2018), and finally,
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- Ozone can remove pollutants in effluents (Brodowska et al., 2018; Nakhate et al., 2019), facilitating the pre-purification of wastewater (Lechuga et al., 2014; Nakhate et al., 2019).

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However, further research is necessary to better understand the properties needed for a specific application, process optimization, and design (Raghunathan et al., 2021).

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In this work, we propose the use of ozonated alkaline formulations (i) to enhance the removal of starch adhered to stainless steel, (ii) to pre-degrade and reduce the toxicity of cleaning wastewater, and (iii) to disinfect the CIP installation against a mature dual-species biofilm formed by *Listeria innocua* and *Pseudomonas putida*, all in a single stage.

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The influence of temperature, time and chemical formulation on the cleaning process has been investigated. This ozone-based CIP process can help develop more efficient, more cost-effective and cleaner industries, using cleaner on-site technologies instead of end-of-pipe treatments, while still meeting regulatory safety standards.

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2. Materials and methods

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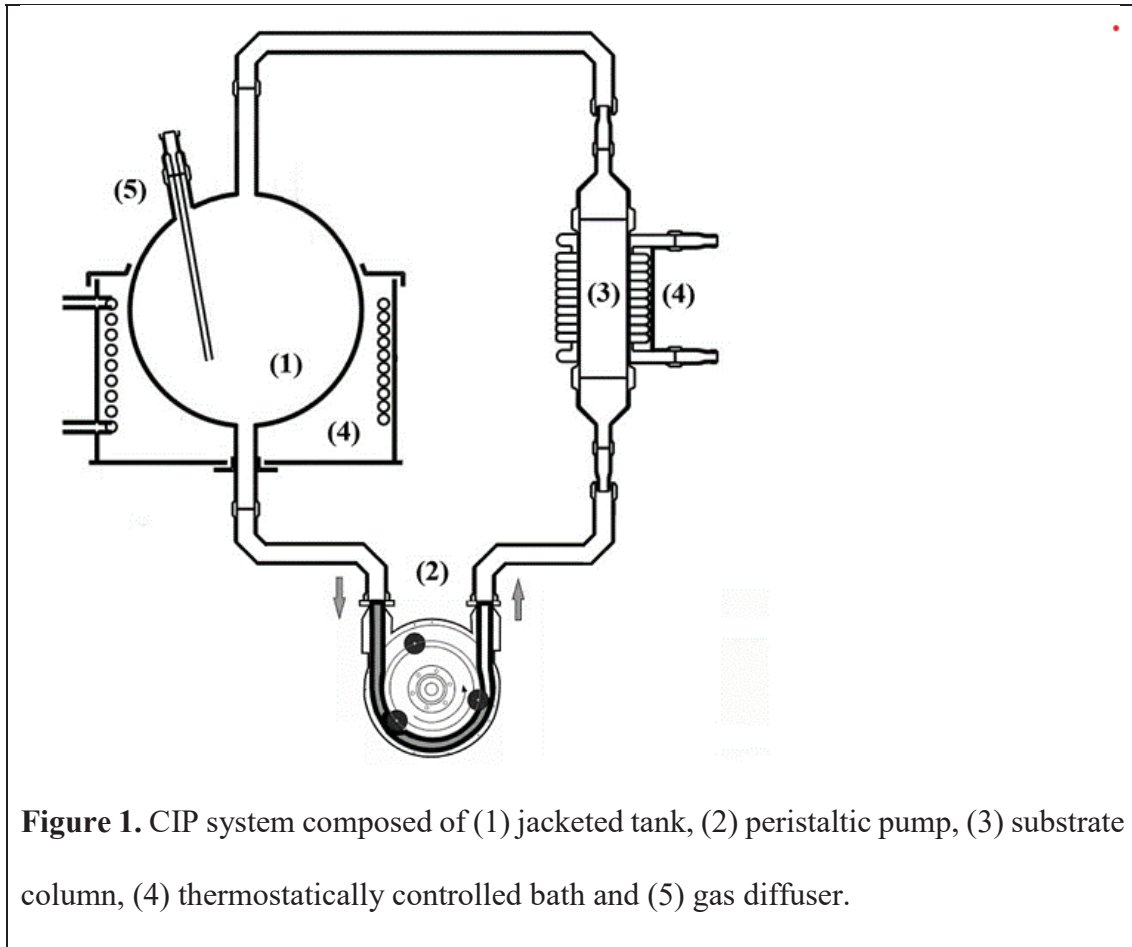
2.1 Substrate, foulant agent, and fouling procedure

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Wads of stainless-steel fibres (weight 0.80 ± 0.01 g, 0.51 mm fibre width, 2.0 ± 0.01 cm sphere diameter) were used as substrate. The substrate was fouled with heat-treated cornstarch as described in Jurado-Alameda et al.(2015a); briefly, 8% w/w cornstarch water suspension (Maizena®) was heated for 1 h at 70°C and cooled at room temperature for 12h. Then, the surface of the wads was impregnated with gel by submersion. The soiled wads were place on a grate, and dried at 60°C for 24 h. Finally, the fouled and dried wads were weighed. The composition of dried starch was 90.37% carbohydrates, 0.42% fat, 0.37% protein, 0.99% ashes, 7.84% humidity. Eight wads were used per cleaning test (2.0 ± 0.1 g of total dried starch mass).

2.2 CIP system

Starch removal was analysed in a simulated CIP system (**Fig. 1**) which allows the modification of the main cleaning factors (i.e. temperature, composition of the solution, substrate, dirt, and flow rate). The CIP device is composed of (1) a jacketed tank that contains the cleaning solution (1 L), (2) a peristaltic pump that supplies an upward flow rate of 60 L h⁻¹ (unless otherwise stated) through a silicone tube (60 cm length, 7 mm inner diameter), (3) a column (8.5 cm height, 2.5 cm diameter, 50 mL) where the fouled substrate is placed with an outlet silicone tube (60 cm long, 7 mm inner diameter) through which the cleaning solution returns to the jacketed tank., (4) a temperature-controlled water bath and (5) a gas diffuser where the oxygen-ozone stream diffuses towards the solution. The ozone generator (Anseros Peripherals COM-AD, Germany) produces ozone by an oxygen stream at a volumetric flow of 40 NL h⁻¹. The ozone concentration was measured (Ozomat GM-6000-PRO, Anseros, Germany) and varied according to the corresponding cleaning test.



2.3 Cleaning method

NaOH aqueous solutions (pH=13.2) were used as a cleaning agent to study the effect of (i) temperature (20-60°C), (ii) ozone concentration (0.00-42.30 g Nm⁻³), and (ii) time (30-120 min) on the removal of starch. Before each experimental assay, ozone flows into the cleaning formulation for 20 minutes. Then, the fouled spheres were introduced in the CIP column. Once the cleaning assay was initiated, 3 mL samples were taken periodically (30-120 min) from the CIP system to evaluate detergency (DePS) by the phenol-sulfuric method (section 2.4) as a function of time. The residual oxidants were eliminated by adding 0.2 mL of Na₂SO₃ (0.2 g L⁻¹) per mL of sample to avoid post-cleaning reactions. Once the cleaning process was finished, spheres were taking out from the CIP column and placed in an oven (60°C, 24h). Detergency (De, %), expressed as the weighing

1 difference of the spheres before and after cleaning determines the efficiency of the
2 cleaning process. Finally, the CIP system was rinsed three times with water (50°C, 5 min).
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4 Samples of cleaning wastewater were also collected, to which 0.2 mL of Na₂SO₃ (0.2 g
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6 L⁻¹) was added per mL of sample, and frozen (-20°C) for further use (toxicity and
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8 biodegradability assays).
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11 The effect of surfactant addition (1.00 g L⁻¹) on starch removal was also studied. The
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13 surfactants tested were: (i) an ionic surfactant, **LAS** (linear alkylbenzene sulfonate, CMC
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15 (37 °C) 1.018 g L⁻¹ (Martínez-Gallegos, 2005), Petresa, Spain); two nonionic surfactants,
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17 (ii) **APG** (Glucopon 650[®], alkylpolyglucoside, CMC (37 °C) 0.073 g L⁻¹ (Bravo et al.,
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19 2005), Henkel, Germany) and (iii) **FEA** (Findet 1214N23[®], fatty ethoxylated alcohol,
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21 CMC (37 °C) 0.021 g L⁻¹ (Martínez-Gallegos et al., 2011), Kao Corporation S.A., Spain)
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23 and an (iv) amphoteric surfactant dependent of pH, **AOX** (Oxidet DM-20[®], lauramine
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25 oxide, Kao Corporation S.A., Spain). Detergency was evaluated at 45 min using NaOH
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27 aqueous solution (pH 13.2) and a 40 NL h⁻¹ oxygen-ozone flow with an ozone
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29 concentration of 42.30 g Nm⁻³.
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39 **2.4 Total soluble carbohydrates content**

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41 Gottschalk et al. (2019) have correlated the online measurement of particle size
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43 distributions during starch cleaning in an automated laboratory test rig, but it is very
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45 difficult to define a correlation when cleaning is due to chemical and physical effects, as
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47 occurs in the present work where ozone is incorporated. In this work, the total
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49 carbohydrates content was analysed by the colorimetric phenol–sulfuric method (DuBois
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51 et al., 1956): 1 mL of cleaning solution (0-120 min) was added to 1 mL of H₂SO₄ (2 N)
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53 and placed in a digester (Spectroquant TR320, Merck) (100 °C, 30 min). After starch
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55 hydrolysis, vials were cooled with ice. Then, 0.10 mL of the sample was diluted up to
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1 5 mL with distilled water. From this volume, 0.5 mL was added to 0.5 mL of phenol 5%
2 (w/v) and 2.5 mL of H₂SO₄ (95%), being stirred and cooled at room temperature. At 15
3 minutes later, the absorbance of the samples was determined spectrophotometrically at
4 490 nm (Cary 100 Bio UV–Visible, Varian). A calibration line, made with D-glucose
5 anhydrous (PA, Panreac), was used to evaluate the starch concentration, considering a
6 conversion factor of 0.9, which defines the stoichiometric relation between starch and
7 glucose (Jaiswal and Prakash, 2013). The detergency using the phenol-sulfuric method
8 (DePS, %) was evaluated by dividing the mass of starch in solution by the initial mass of
9 starch adhered to the substrate. The ozonization process chemically modifies the structure
10 of the starch: the phenol-sulfuric method, which allows calculating DePS, evaluates the
11 soluble sugars but they show different absorptivity depending on their structure.
12 Therefore, the DePS value allows us to check the changes in the chemical composition
13 that the original starchy dirt may have undergone after the ozonation.

2.5 Ozonation of surfactant solution

34 Ozonation (42.30 g Nm⁻³) of FEA (1.0 g L⁻¹) aqueous solution was studied at pH 13.2 and
35 40°C using the CIP device described in **section 2.2**. Samples were extracted at different
36 reaction times (0-120 min), of which residual oxidants were eliminated by adding 0.2 mL
37 of Na₂SO₃ (0.2 g L⁻¹) per mL of sample. Surfactant concentration was determined by the
38 Iodine-Iodide method, a semi-specific method for nonionic surfactants (Jurado et al.,
39 2002) based on the formation of a colored complex between the surfactant and the
40 reagent. The colored complex was determined spectrophotometrically at 500 nm (Cary
41 100 Bio UV-Visible, Varian), where the surfactant concentration and absorbance were
42 proportional. Iodine-Iodide reagent was prepared dissolving 1.00 g of iodine (Panreac)
43 and 2.00 g of IK (Panreac) in 100 mL of water. Then, 0.25 mL of iodine-iodide reagent
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1 was added to 10 mL of sample, stirred, and kept at room temperature for 5 minutes. This
2 colorimetric method was not sensitive to sulfite. The calibration line was made by 1.0 g
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4 L⁻¹ FEA solution at pH 13.2. As surfactants are commonly used for cleaning, this section
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6 analyses the effect of both the surfactant presence in ozone-based basic formulations and
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8 the type of surfactant on the heat-treated starch removal.
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14 2.6 Biofilm removal and disinfection

16 The disinfection and removal process of a mature biofilm adhered to stainless steel was
17 investigated using three different aqueous solutions, NaClO 1%, alkaline solution (NaOH
18 aqueous solution, pH 13.2), and an ozonated alkaline solution (NaOH aqueous solution,
19 pH 13.2) in the CIP system. The methodology followed for biofilm formation and
20 enumeration of biofilm cells is detailed in Medina-Rodríguez et al. (2020). The mature
21 biofilm was formed by *Listeria innocua* (Ref. CECT 910) and *Pseudomonas putida* (Ref.
22 DSM 12264) (obtained from CECT (Spanish Type Culture Collection) and DSMZ
23 (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures).
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26 **To quantify the viable biofilm cells**, biofouled spheres were gently washed with sterile
27 saline solution to remove unattached cells. Then, each sphere was placed into 3 mL of
28 sterile saline solution whereby shaking vigorously for 30 s, adhered cells were removed
29 from the surface. To determine the number of viable cells on the biofilm, serial decimals
30 dilutions of the cell suspension were made and plated on different culture media. *L.*
31 *innocua* (COMPASS Listeria) was incubated at 37°C and *P. putida* (CFC Agar) at 30°C.
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33 Finally, plates were incubated for 48 h before colony counting. When it was not possible
34 to quantify the bacteria below the detection limit ($< 0.3 \log \text{CFU mL}^{-1}$), an investigation
35 was carried out by pre-enrichment in TSB (Biokar Diagnostics) expressing the results as
36 presence or absence.
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1 To assess the **disinfection of the cleaning wastewaters**, samples were extracted during
2 cleaning at different times (10-30 min), analysing the concentration of microorganisms.
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4 Residual ozone was eliminated by adding 0.2 mL of Na₂SO₃ (0.2 g L⁻¹) per mL of sample.
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6 The **disinfection level of the substrate** was studied by evaluation of the survival
7 percentage. Eight biofouled spheres are placed into the column of the CIP device for each
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9 cleaning assay (10-30 minutes, 40°C and 60 L h⁻¹). When the cleaning process was
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11 finished, the spheres were collected and the number of viable biofilm cells of *L. innocua*
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13 or *P. putida* was determined as mentioned above. Lastly, the CIP device was disinfected
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15 by recirculating NaClO solution (1%) for 10 min and rinsed three times with distilled
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17 water (50°C, 5 min).
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26 2.7 Chemical oxygen demand method (COD method)

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29 COD was analysed according to Avila-Sierra et al.(2020). The samples (1.5 mL) were
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31 periodically (30-120 min) withdrawn from the tank during both, cleaning (section 2.3)
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33 and surfactant degradation assays (section 2.5). In this method, 1.5 mL of sample was
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35 added to 1.5 mL of distilled water and mixed with the reagent solution. The reagent
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37 solution was formed by mixing 0.08 g Hg₂SO₄ (Panreac), 4 mL of sulfuric acid reagent
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39 (10 g L⁻¹ SO₄Ag₂ dissolved in H₂SO₄), and 1 mL of 0.5 N K₂Cr₂O₇ aqueous solution. The
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41 vials were shaken and placed into a digester (150°C, 2h). After digestion, the vials were
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43 cooled down to room temperature. Absorbance was measured at 620 nm. The COD
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45 measurements were obtained by a calibration curve at different concentrations of a 0.85
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47 g L⁻¹ KC₈H₅O₄ solution (Panreac, Spain). The Na₂SO₃ used to remove the residual
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49 oxidants did not interfere with COD determination. COD depends on the amount of dirt
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51 present in the cleaning wastewater and its chemical composition after ozonation. To
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53 compare cleaning protocols where different levels of detergency were obtained and
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1 evaluate if the ozonation reduced COD, the COD / De ratio in $\text{mg O}_2 \text{ L}^{-1} \text{ mg starch}^{-1}$ is
2 used to analyse the effect of ozone on the degradation of cleaning wastewater.
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7 **2.8 Biodegradability evaluation (BOD method)**

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9 BOD₅ was measured using respirometry Oxitop Control[®] (WTW, Weilheim, Germany)
10 by monitoring manometric pressure changes when oxygen was consumed to transform
11 organic carbon into CO₂. BOD₅ calculation was based on the assumption that the partial
12 pressure of oxygen in the bottle is at least 60 hPa after the experiments. The solutions
13 used were: **Solution A:** 8.5 g L⁻¹ KH₂PO₄, 21.75 g L⁻¹ K₂HPO₄, 33.4 g L⁻¹ Na₂HPO₄ ·
14 2H₂O, and 0.5 g L⁻¹ NH₄Cl.; **Solution B:** 22.5 g L⁻¹ MgSO₄·7H₂O; **Solution C:** 27.5 g L⁻¹
15 1 CaCl₂; and **Solution D:** 0.25 g L⁻¹ FeCl₃·6H₂O (all reagents were supplied by Panreac,
16 Spain). For biodegradation assays, 100 mL of the cleaning solution **withdrawn** from the
17 **tank** at 30, 60, 90, and 120 minutes, were introduced into bottles (500 mL each), where
18 pH was adjusted to 7 by adding 1 M NaOH and 1 N HCl. Then, 1mL of A and 0.1 mL of
19 each of the solutions (A, B, C, and D) were added with 0.05 mL of sewage water that
20 contains the inoculum. The inoculum used was obtained from the secondary effluent of a
21 municipal wastewater treatment plant that operates with active sludges (Granada, Spain,
22 37°09'54.1"N–3°37'31.8"W). Each bottle was provided with NaOH pellets (Panreac,
23 Spain) for CO₂ absorption. The bottles were placed in a stirring system and incubated at
24 25°C for 5 days in darkness to avoid the production of photosynthetic algae oxygen. As
25 mentioned previously, Na₂SO₃ aqueous solution was used to remove residual ozone.
26 BOD₅ values were recalculated to eliminate the contribution of sulfite. **The BOD₅ / De**
27 **ratio in $\text{mg O}_2 \text{ L}^{-1} \text{ mg starch}^{-1}$ was calculated to compare cleaning protocols where**
28 **different levels of detergency were obtained and evaluate if the ozonation process affected**
29 **the cleaning wastewater biodegradability.**
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2.9 Toxicity evaluation

Toxicity measurements were performed with a LUMISTox® test (UNE-EN ISO 11348-2: 2009), using marine luminescent bacteria *Vibrio fischeri* (NRRL-B-11177, Dr LANGE). Toxicity was based on the bioluminescence inhibition after a certain exposure time with toxic substances, in this case, cleaning wastewaters. The luminescent bacteria, dehydrated and frozen at $-18\text{ }^{\circ}\text{C}$, were reactivated with the suspension supplied by Dr. Lange (Dr. Bruno Lange GmbH & Co., Düsseldorf, Germany) according to detailed in Lechuga et al.(2016). The assay conditions were pH 7.0, 2% NaCl, all the measurements were duplicated for an incubation time of 15 and 30 minutes. 0.5 mL of the 2% NaCl solution (Panreac, Spain) was used as control. At 30, 60, 90, and 120 minutes, samples of the cleaning solution (1.5 mL) were extracted from the tank (fitted to $\text{pH } 7.0 \pm 0.2$ with 1 M NaOH / 1 N HCl) and were added to 0.5 mL of the reactivated bacteria solution to measure the toxicity of cleaning wastewater. The luminescence was determined at $t = 0$ min and for incubation times of 15 and 30 minutes. All measurements were performed twice. The Toxicity / De ratio in % inhibition mg starch^{-1} was calculated and allowed to compare cleaning protocols where different levels of detergency were obtained and analyse the effect of ozone on the Toxicity of cleaning wastewater.

3. Results and discussion

3.1 Starch removal using ozone-based cleaning formulations

The disinfecting effect of ozone is well known but its potential use as a cleaning agent has barely been investigated. Vicaria et al.(2017) showed that the cleaning of cornstarch was practically negligible below pH 10, requiring the use of stronger alkaline conditions

(Vicaria et al., 2017). In this section, the incorporation of ozone and surfactants in alkaline formulations is analysed concerning starch cleaning.

3.1.1 Ozonated alkaline solutions

De and DePS were evaluated using NaOH aqueous solutions (pH 13.2) as a function of time (0 – 120 minutes), temperature (20, 40, 60°C) and ozone concentrations (0.00, 21.15, 42.30 g Nm⁻³) (Fig. 2).

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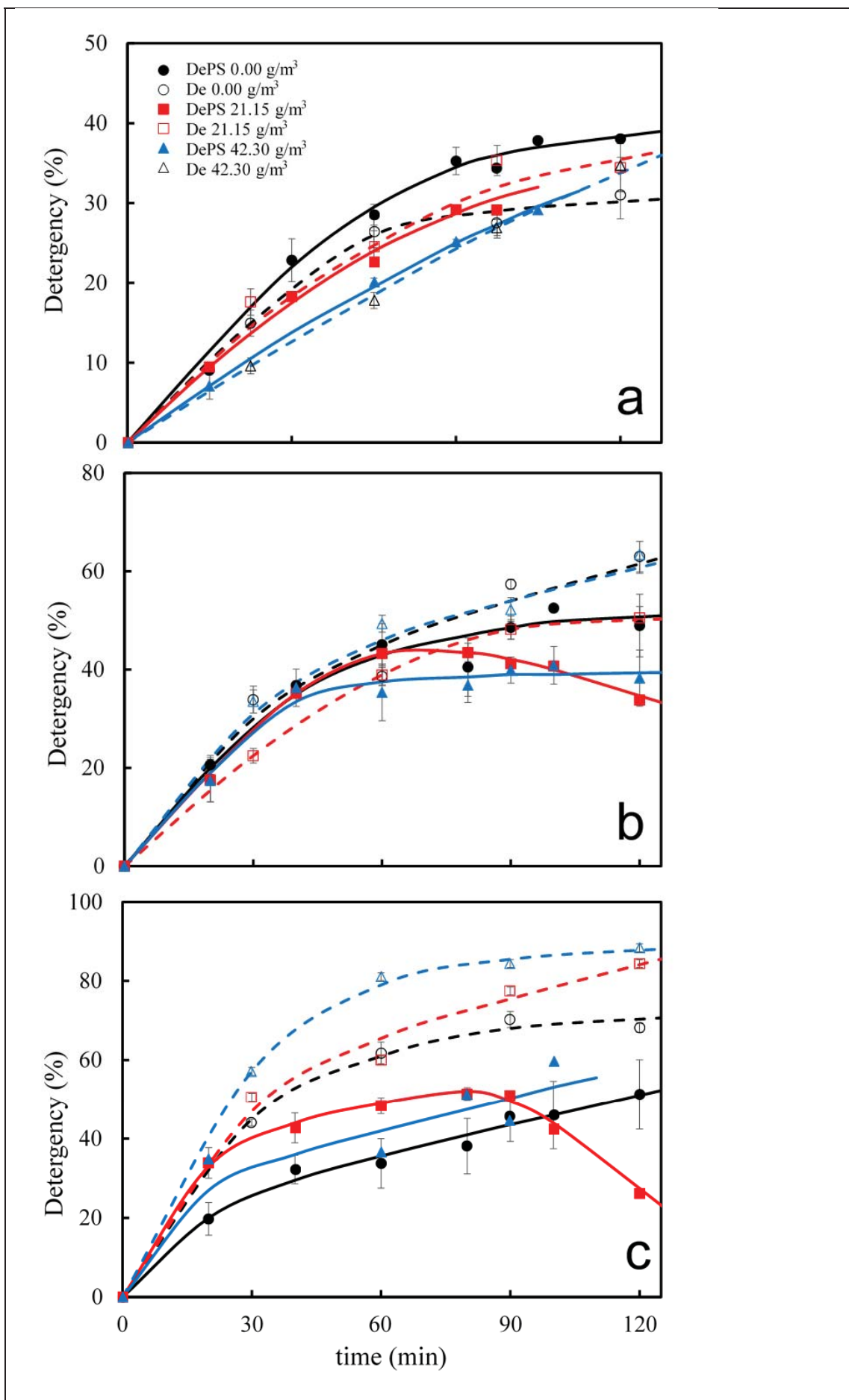


Figure 2. Detergency (De and DePS) as a function of ozone concentration (0.00, 21.15, 42.30 g Nm⁻³) and cleaning time (0-120 minutes). Influence of temperature: a) 20°C, b) 40°C, c) 60°C. CIP parameters: recirculation flow 60 L h⁻¹, oxygen-ozone flow 40 NL h⁻¹. Lines are a guide to the eye: continuous line for DePS and dashed line for De. Error bars show ±SD of at least two measurements.

The starch removal was enhanced as cleaning temperature, time, and ozone concentration increased. In absence of ozone, De roughly doubled its value as temperature increased from 20 to 60°C, reaching 31.5% and 68.2% at 120 minutes respectively (Fig. 2). This ability of strong alkaline solutions to remove starch can be explained by the changes produced in the structure of starches (Han and Lim, 2004) and by the negative charge that stainless steel and starches have on basic media, where electrostatic repulsion might facilitate cleaning (Otto et al., 2016). When ozone was incorporated into the cleaning formulation, detergency was highly dependent on both ozone concentration and cleaning temperature. At 60°C and 120 minutes, De was favoured as ozone concentration was raised: an increase of 24% at 21.15 g Nm⁻³ and 30% at 42.30 g Nm⁻³ regarding the absence of ozone. At 120 min and 42.30 g Nm⁻³ of ozone, the highest De values were reached when temperature increased; De increased ca. 83% at 40°C and ca. 155% at 60°C with respect to 20°C. This detergency improvement, mainly at 60°C and 42.30 g Nm⁻³ of ozone, might be related to several interconnected factors:

- (i) the starch chemical modifications that ozonation produces. For instance, starch properties such as gel strength, apparent viscosity, and swelling are modified after ozonation and gelatinization (Liu et al., 2016; Castanha et al., 2019b), where cleavage of glycosidic bonds and the presence of electronegative groups favours solubility, and

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(ii) the formation of non-selective radicals more oxidizing than ozone (Boczka et al., 2017), especially at high pH values and higher temperatures, where the ozone decomposition rate and the radicals' production are favoured (Brodowska et al., 2018).

The lower value of DePS vs. De at high temperature indicated that the starch was chemically modified. This chemical modification seems to favor the removal of starch from the surface, hence higher De values were obtained. The cleaning efficiency based on the concentration of soluble carbohydrates in the cleaning solution (DePS; section 2.4) has been also analysed:

- At 20°C (Fig. 2a), DePS slightly decreased as ozone concentration increased, showing similar De and DePS levels.
- At 40°C (Fig. 2b), differences between De and DePS can be observed as a function of ozone concentration, especially above 40 minutes: at 21.15 g Nm⁻³, there was a drastic DePS decrease, whilst De remained practically constant up to 120 minutes. At 42.30 g Nm⁻³, the De-DePS difference is further increased from 40 minutes onwards, remaining DePS constant whilst De increases.
- But the most pronounced differences between De and DePS were observed as both ozone and temperature increased. In absence of ozone and 60 °C (Fig. 2c), DePS was 40 % lower than De at 60 minutes. When ozone was incorporated, the differences between De and DePS at 40-100 min were 35-50 % and 40-50 % for 21.15 and 42.30 g Nm⁻³, respectively.

These observations could be explained assuming that (i) strong alkaline media and (ii) the formation of ozone radicals vary the sugars formed during the process of cleaning/oxidation, as the oxidants react with α -1,4-D-glucose chains of amylose and/or amylopectin polymers forming carbonyl and carboxyl groups, limiting subsequently its

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quantification by phenol-sulphuric method (**section 2.4**). Previous works demonstrated how ozonation leads to important structural changes of starches, replacing hydroxyl groups with carboxyl and carbonyl groups, and cleavage the glycosidic bonds of both amylose and amylopectin molecules, decreasing starch molecular size (Castanha et al., 2017, 2019a). In addition, hydroxyl radicals have also been found to react with poly and oligosaccharides breaking the glucosidic bonds (Dai, et al., 2017). Overall, the reaction rates of ozone-starch and/or ozone-solubilised sugars in alkaline media seem to be dependent on both temperature and ozone concentration.

3.1.2 Addition of surfactants to the ozonated solution

The chemical action during cleaning can be improved using surfactants (Basso et al., 2017) because they favor soil penetration, emulsification, and surface wetting (Moerman et al., 2014). Currently, surfactants of petrochemical origin are being replaced by other more environmentally friendly surfactants such as fatty alcohols or alkylpolyglucosides (Rebello et al., 2014). In order to improve starch removal, four different types of surfactants (1.00 g L⁻¹; **section 2.3**) were added to the ozonated cleaning solution:

- The incorporation of LAS and APG reduced De significantly (**Fig. 3a**) in the range of temperature tested (25-45°C). Although the addition of APG (1.00 g L⁻¹) to pH 13.2 solutions in the absence of ozone improved detergency (Jurado-Alameda et al., 2015a), there was no removal improvement when ozone was added (**Fig. 3a**), which is consistent with Herrera-Márquez et al.(2019). As in the case of LAS, this fact could be due to surfactant removal during ozonation (Jurado-Alameda et al., 2012b) as ozone exhibits affinity for different alkyl chain homologues of LAS (Vicaria et al., 2016).

- **AOX** did not improve either detergency at 25 and 35°C but increased slightly the removal of starch at 45°C. Despite this slight cleaning improvement at higher temperatures, the use of AOX should be discouraged as amines can be transformed into subproducts containing a nitrogen-oxygen bond, and lead to the formation of nitroalkanes during ozonation posing adverse effects on the aquatic environment and human health (Lim et al., 2019).
- Finally, even though **FEA** did not affect De in the absence of ozone (Jurado et al., 2015b), its incorporation to the ozonated pH 13.2 solution (**Fig. 3a**) enhanced cleaning; De increased 27, 43, and 46% at 25, 35 and 45°C respectively when compared with cleaning in surfactant absence. Vicaria et al. (2016) also reported high deterative effectiveness when protocols included ozone and ethoxylated fatty alcohol at 45 ° C. Thereby, attending to the promising synergetic effect of ozone and FEA, surfactant degradation was studied measuring both surfactant concentration (**section 2.5**) and COD (**section 2.6**) over time (120 minutes). The concentration and COD of FEA solution decreased as the reaction time increased (**Fig. 3b**): there was a 50% reduction of the initial surfactant concentration during the first 40 minutes, whilst COD also decreased linearly by reduction of the number of oxidizable pollutants found in the surfactant-based cleaning formulation. Therefore, although the surfactant improved the removal of starch adhered to stainless steel, its rapid degradation at relatively short cleaning times could limit its use.

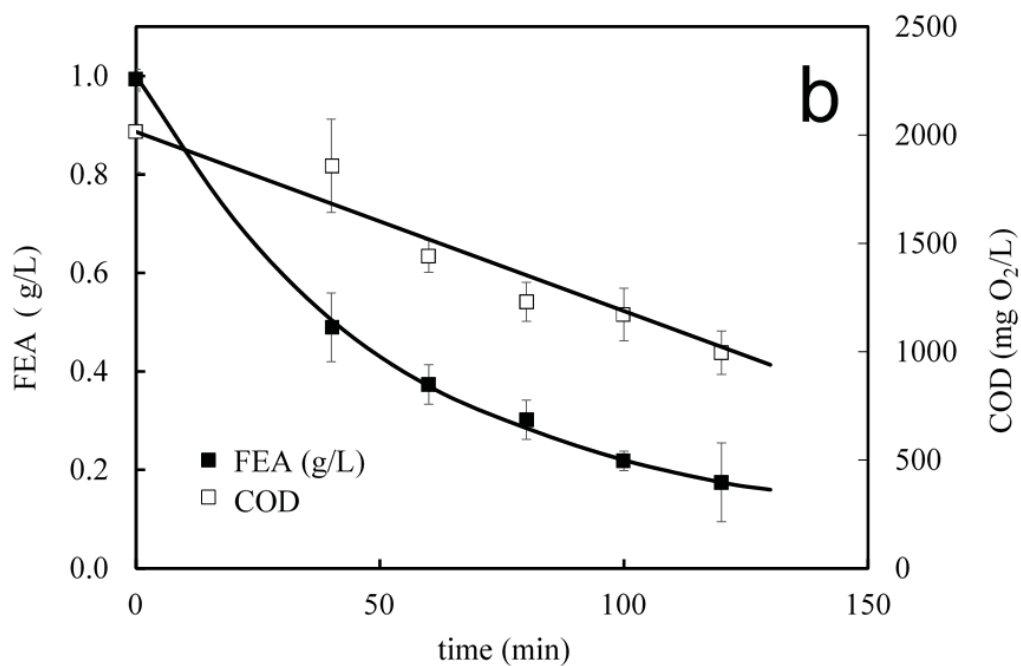
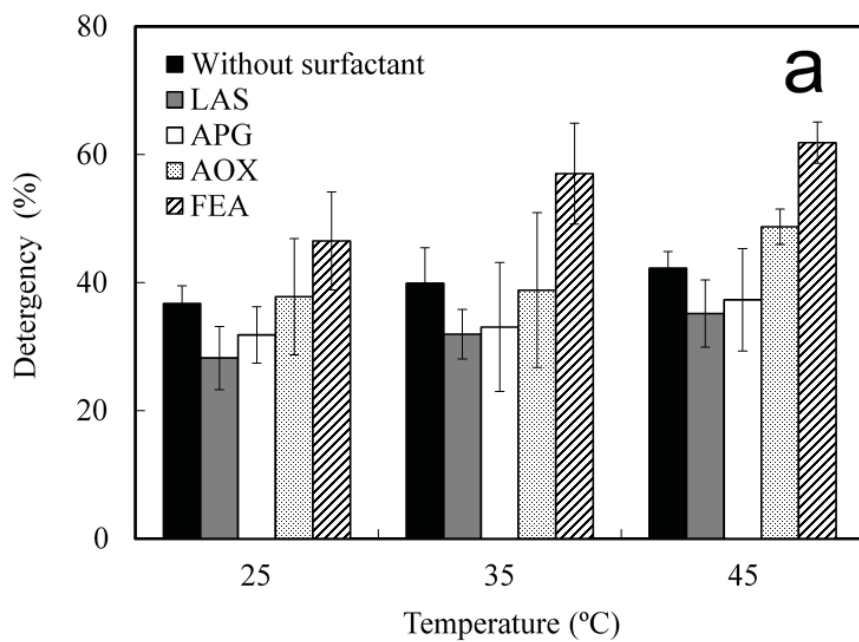


Figure 3. Surfactants addition on ozone-based cleaning formulations. (a) Influence of surfactants and cleaning temperature on starch removal (De, %) (85 L h^{-1} , 45 min). (b) Ozonation of FEA aqueous solution: surfactant concentration and COD vs. time. Surfactant concentration 1.00 g L^{-1} , pH 13.2, recirculation flow 60 L h^{-1} , oxygen-ozone

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flow 40 NL h⁻¹, and ozone concentration 42.3 g Nm⁻³. Error bars show ±SD of at least three assays.

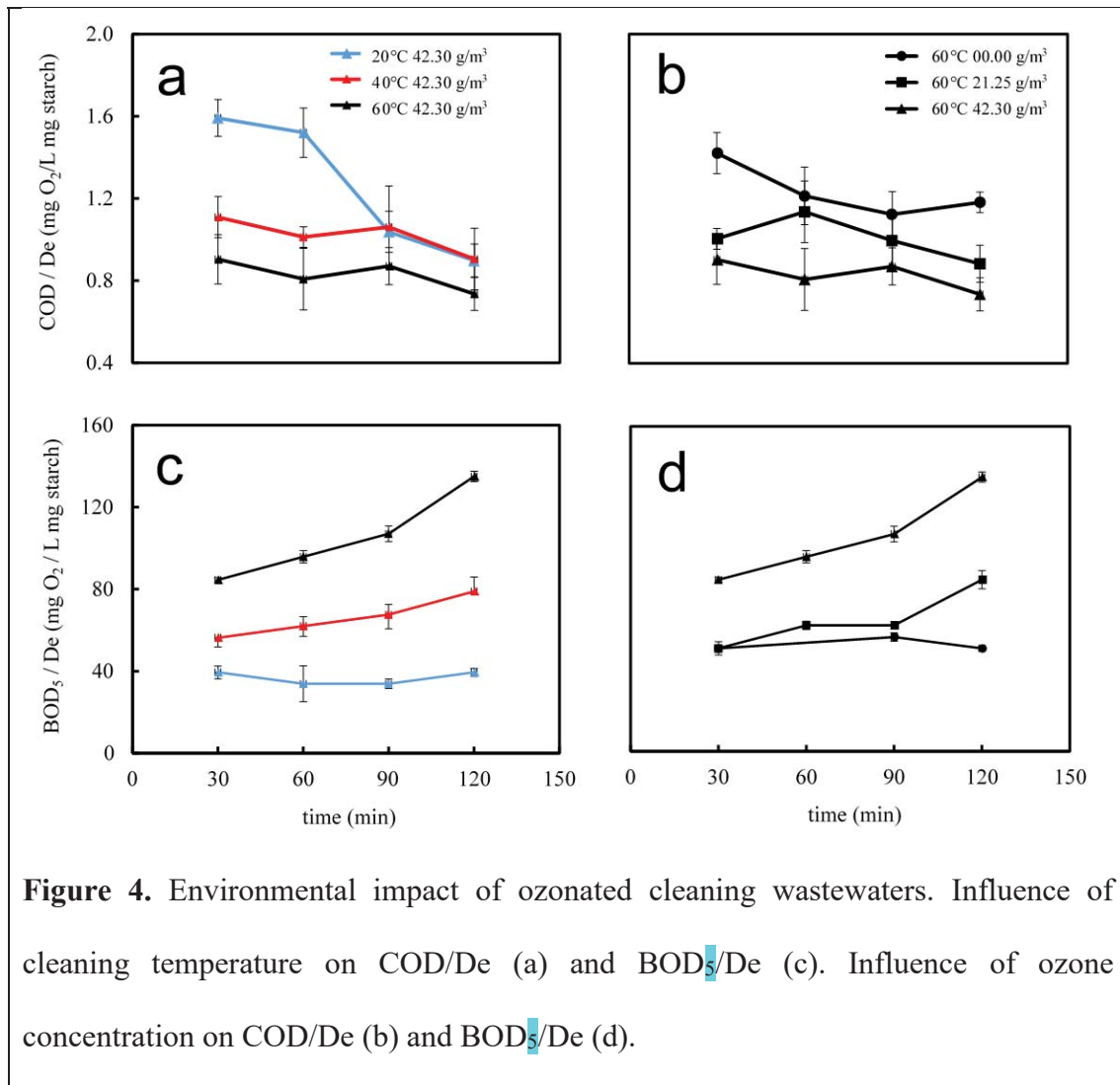
3.2 Environmental impacts of ozonated cleaning wastewaters

As highlighted previously, cleaning products cause wastewater contamination, requiring additional disposal costs. In this section, the environmental impact of ozonated alkaline wastewater was evaluated by measuring the degradation of organic matter (COD and BOD) and toxicity as a function of cleaning temperature (20-60°C), ozone concentration (0-42.30 g Nm⁻³), and time (0-120 minutes).

3.2.1 Chemical oxygen demand (COD)

In the absence or presence of ozone (21.15 g Nm⁻³) the temperature (20-60°C) had no noticeable impact on the COD/De values over time, remaining practically constant at 1.2 ± 0.2 and 1.1 ± 0.1 mg L⁻¹ mg⁻¹ respectively (data not shown). At higher ozone concentration (42.30 g Nm⁻³), an increased temperature reduced the COD/De ratio (Fig. 4a). At 20°C and short cleaning times (< 60 minutes), the COD/De values of 1.6 mg L⁻¹ mg⁻¹ were obtained. At longer times, the COD/De shows similar values to those at 40°C. At higher temperatures, the COD/De ratio decreased slightly over time and was also reduced about 20% from 40 to 60°C (1.0 ± 0.1 and 0.8 ± 0.1 mg L⁻¹ mg⁻¹, respectively). The effect of the ozone concentration is shown at 60°C in Fig. 4b. Although the COD/De values did not change significantly over time, ozone reduced the COD of the cleaning wastewater. Without ozone (except at 30 min with 1.4 mg L⁻¹ mg⁻¹), the COD/De ratio was ca. 1.2 mg L⁻¹ mg⁻¹. The initial COD/De when ozone was introduced diminished ca. 0.2 and 0.4 mg L⁻¹ mg⁻¹ at 21.15 and 42.30 g Nm⁻³ respectively. Therefore, the COD/De

ratio was dependent on two factors, temperature and ozone concentration, and barely affected by cleaning time, especially above 60 minutes.



3.2.2 Biochemical oxygen demand (BOD₅)

An increase in the temperature of the cleaning solution increases BOD₅/De over time. For instance, in **Fig. 4c**, BOD₅/De values were between 33.8-39.4 and 84.5-135.0 mg O₂ L⁻¹ mg⁻¹ at 20 and 60°C respectively, when ozone was introduced (42.30 g Nm⁻³). Similar behaviour was observed as the ozone concentration increased (**Fig. 4d**; data at 60 °C), where higher ozone concentration led to higher BOD₅/De levels. Without ozone (60°C), the BOD₅/De ratio remained practically constant over time. When ozone was introduced

1 (21.15 g Nm⁻³), BOD₅/De values were between 50.7 and 84.5 mg L⁻¹ mg⁻¹, increasing
2 **mainly** at 120 minutes. **M**ore marked differences on BOD₅/De **vs.** time were observed at
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4 higher ozone concentration (42.3 g Nm⁻³); BOD₅/De values were between 84.5 and 135.0
5 mg O₂ L⁻¹ mg⁻¹ starch. Therefore, BOD₅/De also depended **o**n two factors, temperature
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7 and ozone concentration, but now it was significantly affected by cleaning time,
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9 especially above 60 minutes. **The increase of BOD₅/De ratio with raising temperature and**
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11 **ozone concentration implies that the organic matter is more available to the**
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13 **microorganisms, thus improving the cleaning solution biodegradability.**
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22 **3.2.3 Toxicity**

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24 Although all ozone concentrations showed similar behaviour, ozonation at 42.30 g Nm⁻³
25 showed **the** clearest differences between toxicity profiles during cleaning (**Fig. 5a**): higher
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27 temperatures reduced Toxicity/De levels over time, showing the most **notable** differences
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29 between 20 and 40°C. The highest Toxicity/De reduction over cleaning time was
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31 observed at 20°C; it decreased ca. 0.06 % mg⁻¹ from 30 to 120 minutes. At 30 minutes of
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33 cleaning, sample toxicity was slightly higher at 60°C than at 40°C, yet the lowest
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35 Toxicity/De level was reached at 60°C for 60 minutes, remaining practically constant over
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37 time (up to 120 minutes). In most cases, higher inhibition levels were observed at **higher**
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39 incubation periods (30 min).
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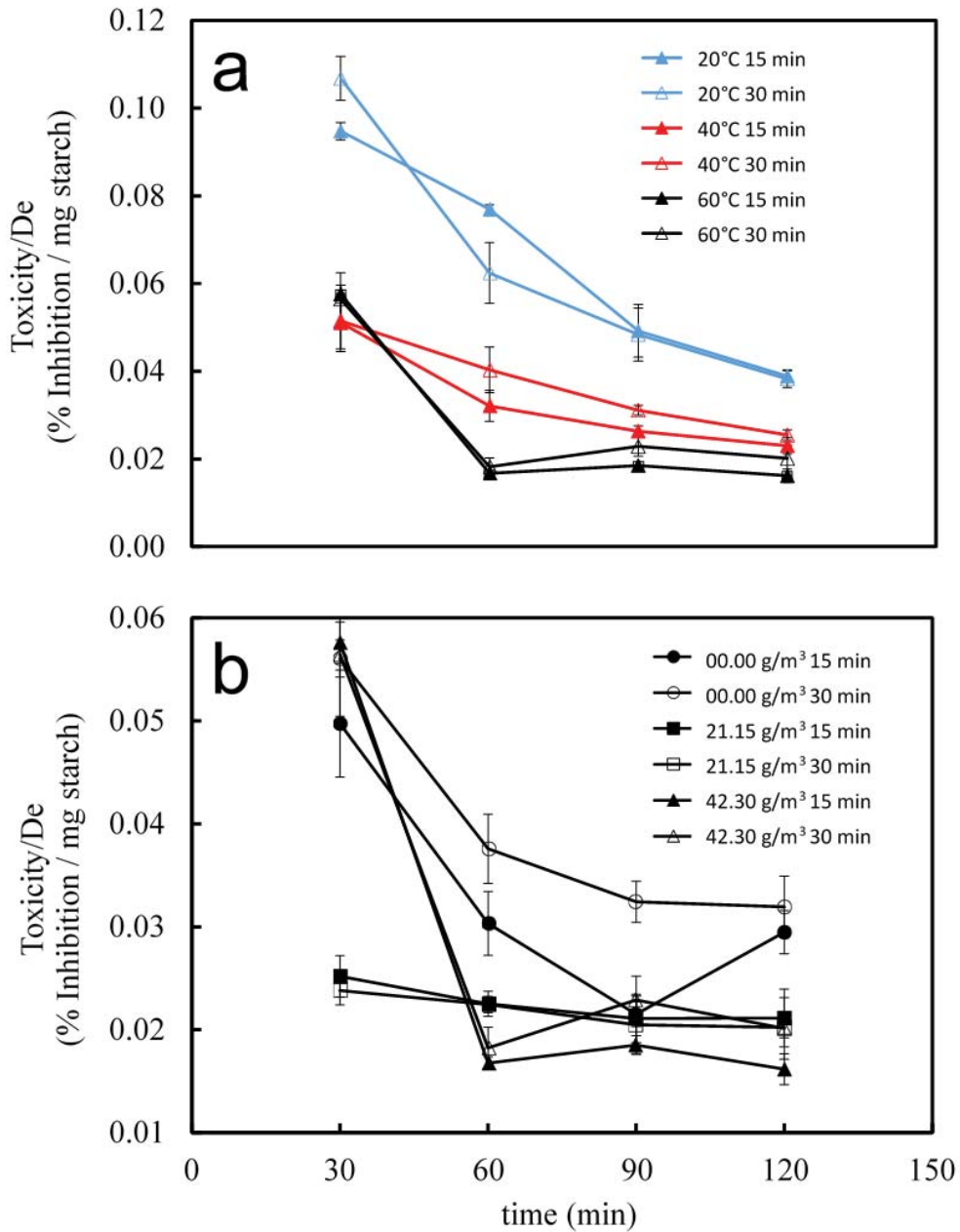


Figure 5. Toxicity/De profiles vs. time of the cleaning wastewater as a function of (a) cleaning temperature (ozone concentration 42.30 g Nm⁻³) and (b) ozone concentration (cleaning temperature 60°C). Toxicity was studied at 15 and 30 minutes.

When the temperature was constant, an increased ozone concentration reduced Toxicity/De (see example at 60°C in Fig. 5b). The Toxicity/De ratio decreased over time from 0.0769 to 0.0360 % mg⁻¹ and from 0.0230 to 0.0202 % mg⁻¹ at 20 and 60°C

1 respectively with ozone (21.15 g Nm⁻³). A similar trend was found at 42.3 g Nm⁻³, where
2 Toxicity/De values decreased from 0.0947 to 0.0383 % mg⁻¹ and from 0.0565 to 0.0162
3 % mg⁻¹ at 20 and 60°C, respectively. The Toxicity/De values were high at 30 minutes
4 (Fig. 5b) using 42.3 g. ozone Nm⁻³, but it led to the most significant reduction and the
5 lowest toxicity levels from 60 to 120 min.
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14 3.3 Disinfection of dual-species biofilm by alkaline formulations

15 Alkaline formulations prevent bacterial colonization (Nostro et al., 2012). This section
16 analyses the efficacy that an alkaline solution, in the absence or presence of ozone, as a
17 disinfectant against a mature dual-species biofilm formed by *Pseudomonas putida* and
18 *Listeria innocua* (section 2.6). The disinfection level was determined on (i) the cleaning
19 wastewater and (ii) the stainless-steel substrate since surface-adhered microorganisms
20 might be more resistant to disinfectants than those in suspension (Chavez de Paz et al.,
21 2007). NaClO 1% solution was used as a control.
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33 The dragging effect of the CIP flow (60 L h⁻¹) was measured by performing a water rinse
34 at 40°C for 30 minutes. The initial inoculum concentration on the substrate, 7.5 ± 0.9 log
35 CFU mL⁻¹ for *Listeria innocua* and 7.9 ± 1.0 log CFU mL⁻¹ for *Pseudomonas putida*, was
36 reduced 1.6 and 1.4 log CFU mL⁻¹ units respectively after rinsing (Table 1). In the biofilm
37 matrix, *P. putida* cells were found to be more resistant to removal than those of *L.*
38 *innocua*, suggesting stronger adhesion strength to the metal surface, especially at short
39 cleaning times (Table 2). No cell inactivation was noticed during rinsing.
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53 **Table 1.** Effect of different disinfection treatments on stainless steel. Experimental
54 results, obtained at 40°C and 30 min, are expressed as log CFU mL⁻¹. Experiments with
55 pre-enrichment of the culture medium were performed in cases where the detection limit
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was less than 0.3 log CFU mL⁻¹, and results are expressed as presence or absence. NaClO 1% solution is used as a control.

Strain	Initial inoculum	Water rinsing	NaClO 1%		NaOH solution (pH 13.2)		NaOH solution (pH 13.2) + ozone (42.3 g Nm ⁻³)	
<i>L. innocua</i>	7.5 ± 0.9	5.9 ± 0.3	< 0.3	Absence	< 0.3	Absence	< 0.3	Absence
<i>P. putida</i>	7.9 ± 1.0	6.5 ± 0.9	< 0.3	Absence	< 0.3	Absence	< 0.3	Absence

Table 2. Evolution of the viability of *L. innocua* CECT 910 and *P. putida* DSM 12264 in the cleaning wastewater after disinfection at 40°C and different times, 10, 20 and 30 minutes. Initial inoculum is expressed as log CFU mL⁻¹. Experiments with pre-enrichment of the culture medium were performed in cases where the detection limit was less than 0.3 log CFU mL⁻¹, and results are expressed as presence or absence. NaClO 1% solution is used as a control.

Test	10 min		20 min		30 min	
	<i>L. innocua</i>	<i>P. putida</i>	<i>L. innocua</i>	<i>P. putida</i>	<i>L. innocua</i>	<i>P. putida</i>
Initial inoculum	5.58	2.12	5.82	4.23	5.82	5.08
NaClO 1%	Absence	Absence	Absence	Absence	Absence	Absence
NaOH solution (pH 13.2)	Absence	Absence	Absence	Absence	Absence	Absence
NaOH (pH 13.2) + ozone (42.3 g Nm ⁻³)	Absence	Absence	Absence	Absence	Absence	Absence

Alkaline formulations (pH=13.2, 40°C and 60 L h⁻¹), in the absence or presence of ozone (42.3 g Nm⁻³), produced a total removal of microorganisms on the cleaning wastewater in short times; the microorganism concentration was reduced to < 0.3 log CFU mL⁻¹ at 10 minutes (**Table 2**). NaClO 1% and alkaline solutions were effective on disinfection since no active cells were found (< 0.3 log CFU mL⁻¹) on the stainless-steel substrate (30 min) (**Table 1**). The peptizing action of the alkaline solutions against EPS (extracellular polymeric substances) and their surfactant properties (Sharma et al., 2005) cause high lethality of the biofilm, possibly due to the dissolution of polymeric substances which attach cells to the substrate, and the disruption of cytoplasmic membrane integrity through

1 saponification (Ammor et al., 2004). Unfortunately, the disinfection of the alkaline
2 solution hid the effect of ozone. However, the disinfection level depends strongly on both
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4 the environmental conditions and the microbial species (Yoo, 2009), where biofilm
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6 sensitivity to alkaline stress could be also compromised by ageing (Stopforth et al., 2002).
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8 On this matter, the ozone could have further potential implications such as a higher
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10 efficacy against biofilm without residues (Cabo et al., 2009) avoiding **biofilm** formation
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12 on surfaces treated with ozone, or the formation of hydroxyl radicals on the biofilm
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14 surface that improve the sequential secondary treatment with other disinfectants (e.g.
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16 H₂O₂) (Tachikawa and Yamanaka, 2014). Therefore, alkaline solutions showed an
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18 excellent level of disinfection, making the use of complementary disinfection stages
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20 unnecessary, and saving operational costs and time in CIP systems.
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28 **4. Conclusions**

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30 The effect of alkaline ozonated formulations on the removal of starch adhered to stainless
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32 steel, the environmental impact of cleaning wastewater, **and** the disinfection capacity
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34 against a mature biofilm formed by *Pseudomonas putida* and *Listeria innocua* were
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36 investigated as a function of temperature, ozone concentration, and time. The combined
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38 action of ozone and strong alkalinity improved starch removal as both cleaning time and
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40 temperature increased. The starch structure and composition **were** affected by NaOH-
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42 starch and NaOH-ozone-starch interactions, favouring its solubilization and detergency,
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44 **especially at highest temperature and ozone concentration.** Of the surfactants tested, only
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46 FEA improved starch removal, although the incorporation of FEA into the ozone cleaning
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48 formulation should be limited; more than 50% of the surfactant was degraded after 40
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50 minutes of ozonation. The COD, BOD₅ and Toxicity of the cleaning wastewaters were
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52 highly dependent on the CIP parameters, decreasing the environmental impact **when**
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cleaning temperature and ozone concentration increased, obtaining the best results at the highest temperature, ozone concentration and time tested. Moreover, the use of alkaline formulations in the absence or presence of ozone also led to a total reduction of the microorganisms in the CIP system, both in the wastewater and on the metal surface. Therefore, ozone-based CIP protocols provide a novel way to achieve cleaner and safer industries, improving the fouling removal and disinfection, and reducing the environmental impact in a single step, saving operational cost and time.

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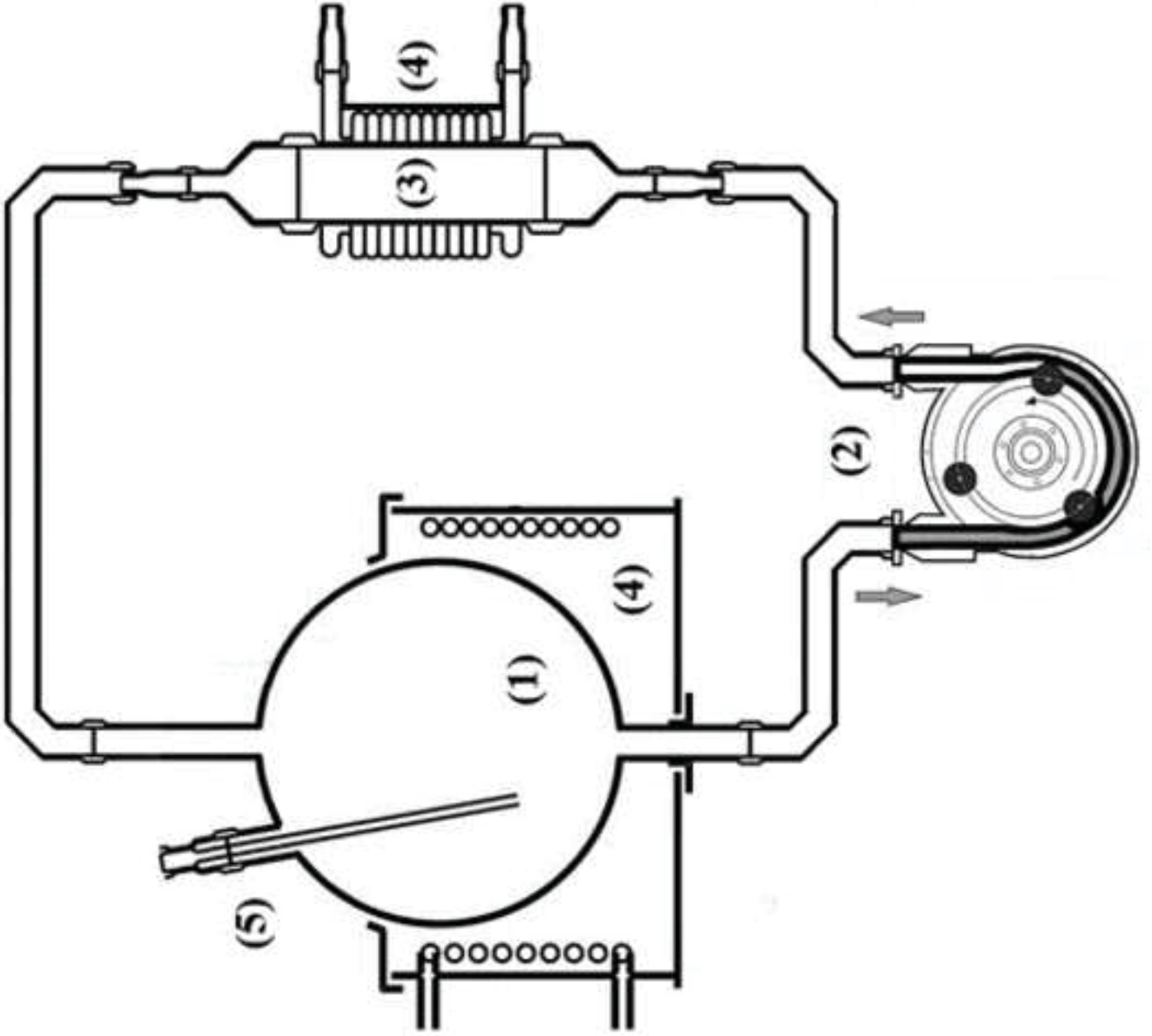


Figure 1

Figure 2

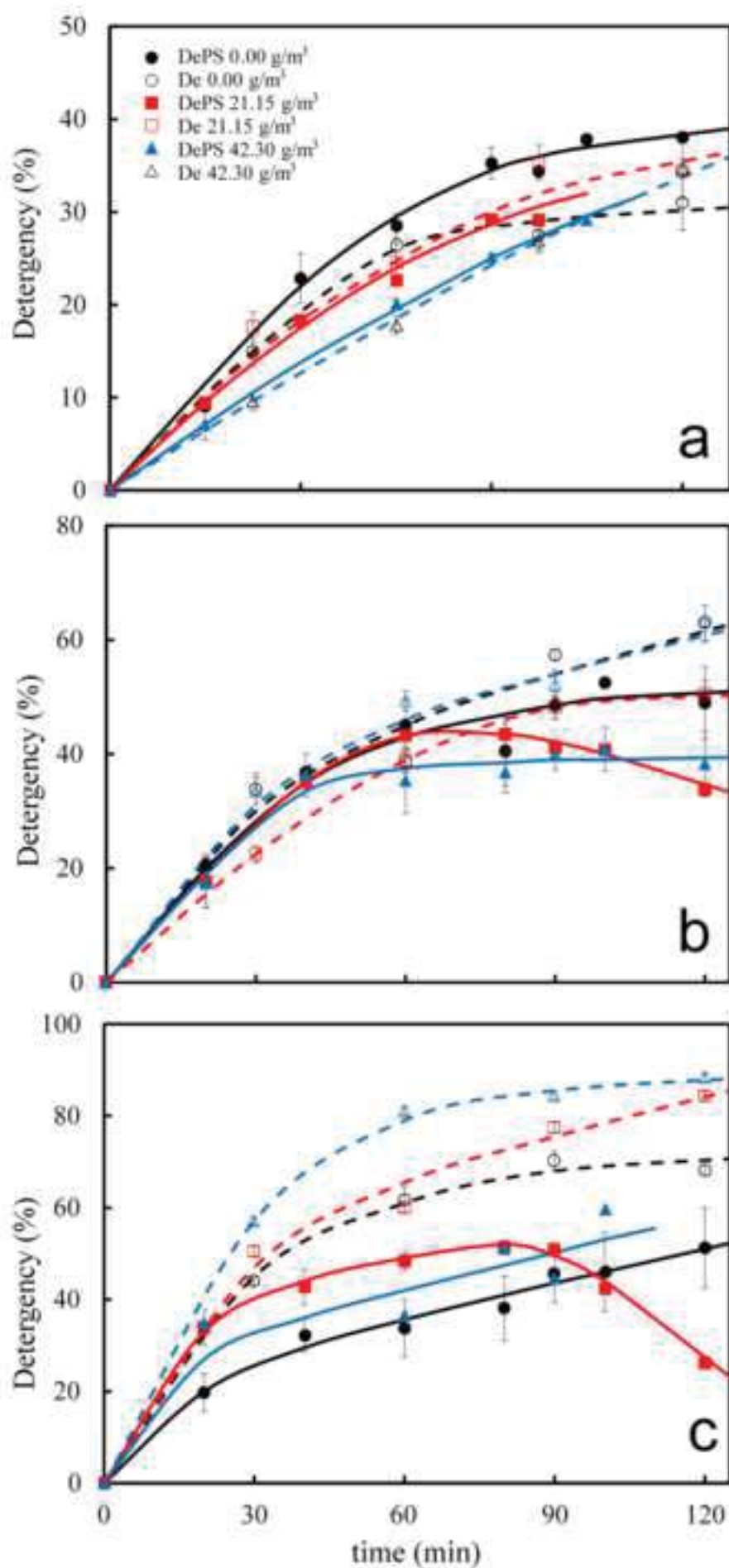
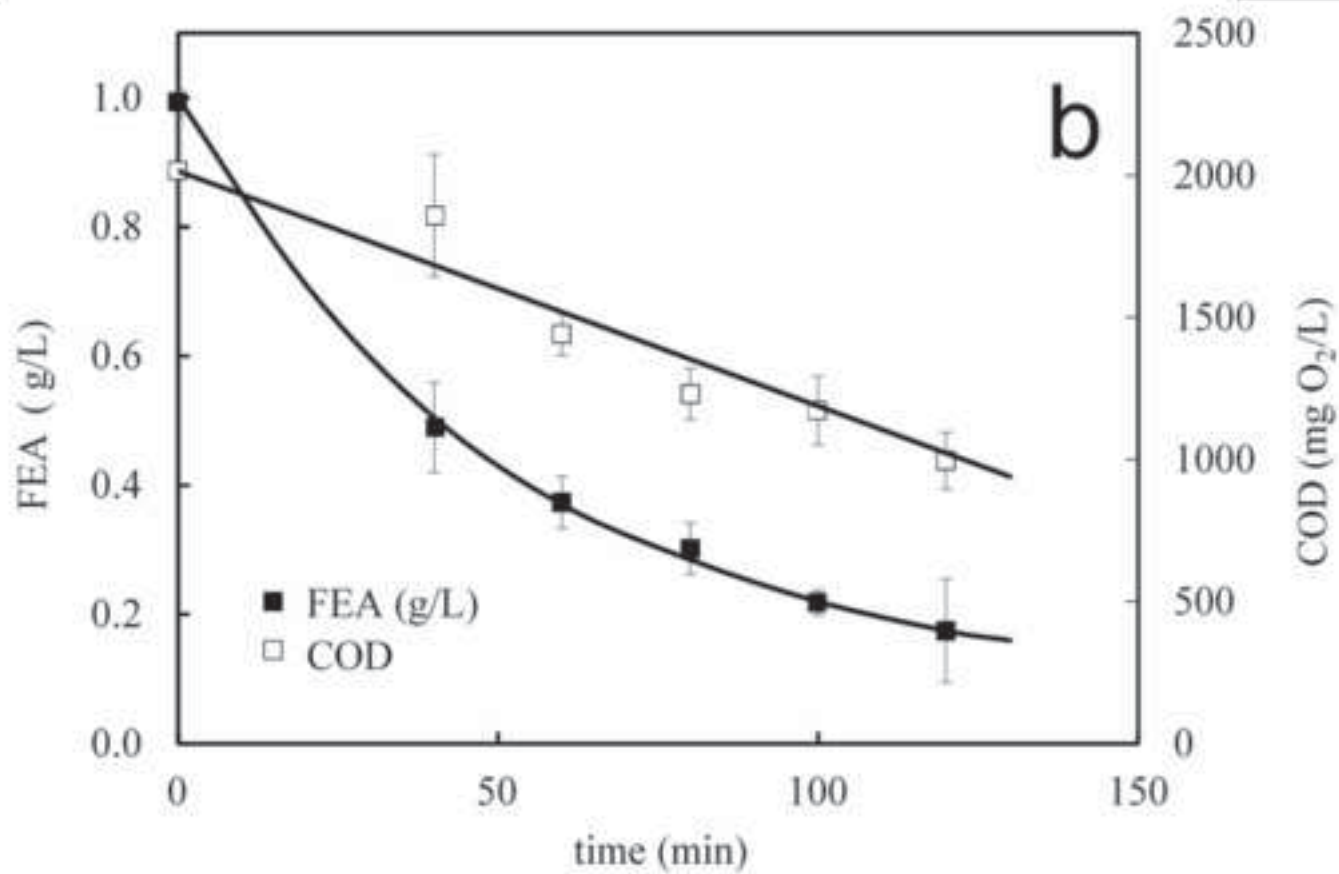
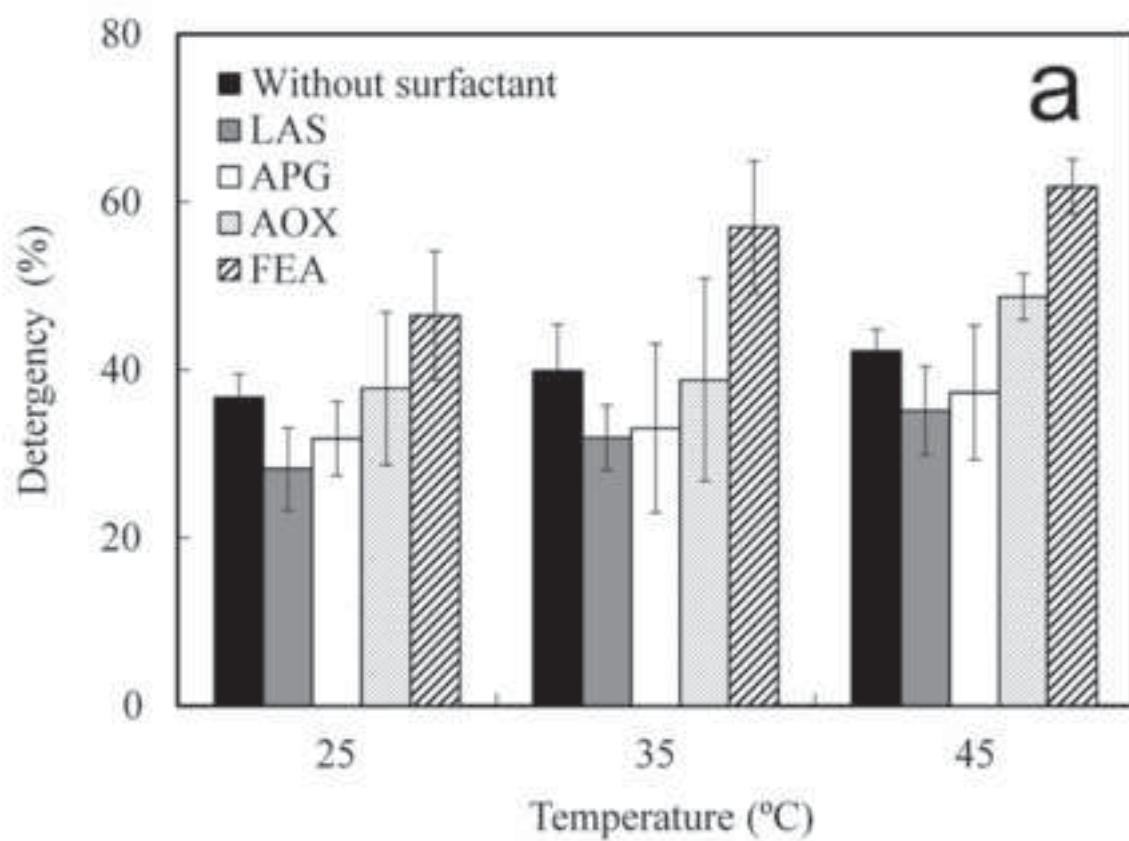


Figure 3



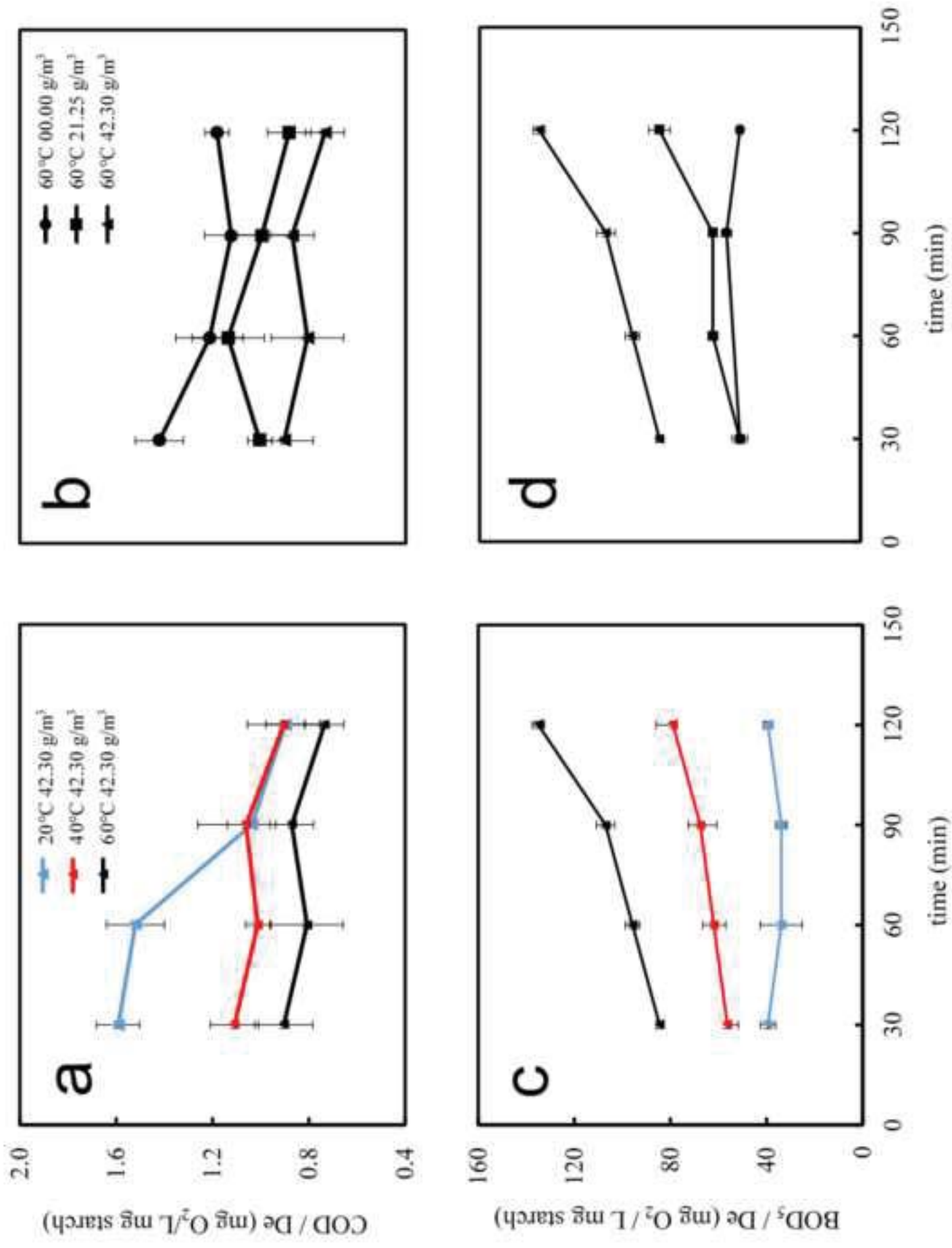
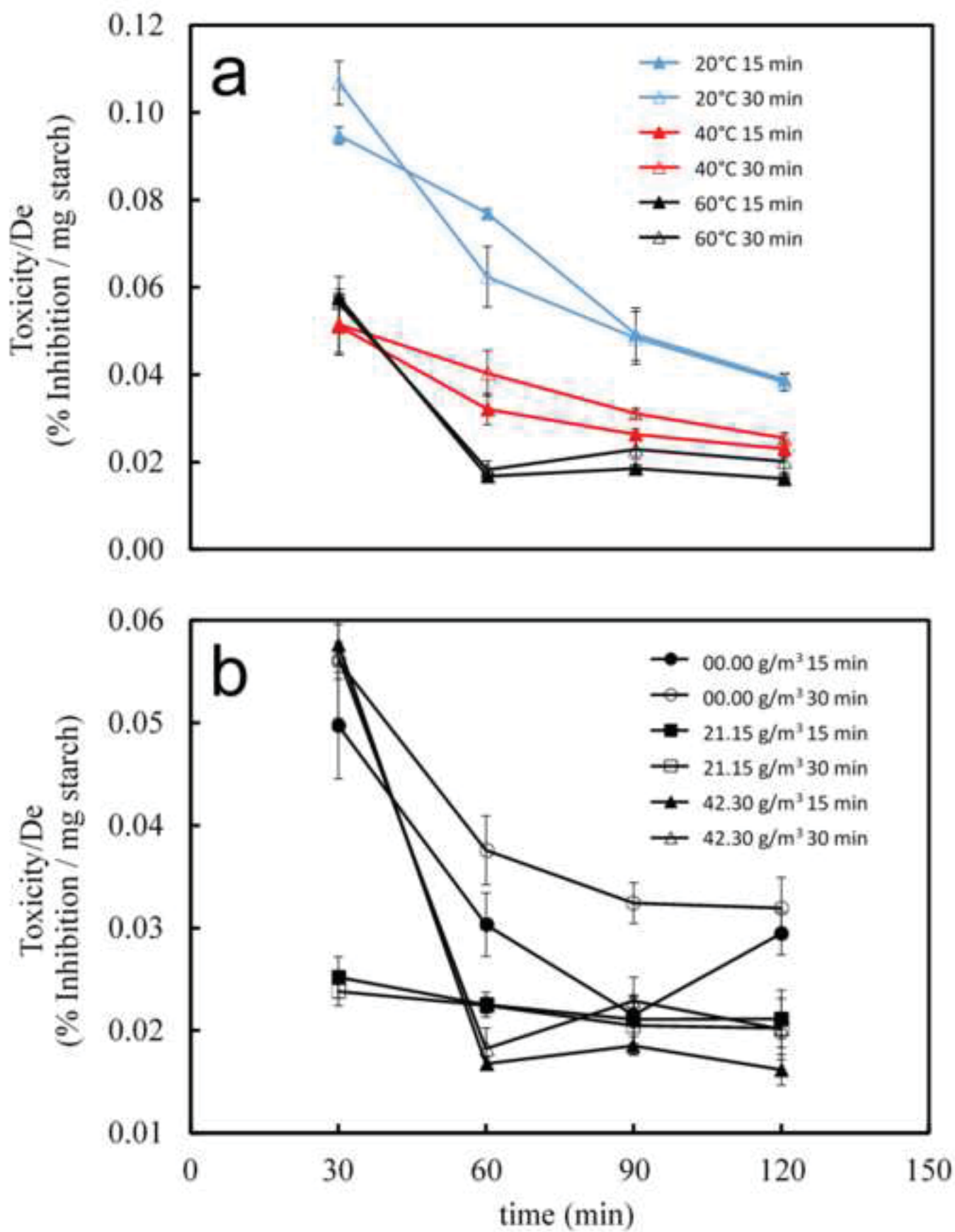


Figure 5



1 **Table 1.** Effect of different disinfection treatment on stainless steel. Experimental
 2 results, obtained at 40°C and 30 min, are expressed as log CFU mL⁻¹. Experiments with
 3 pre-enrichment of culture medium were performed in cases where the detection limit
 4 was less than 0.3 log CFU mL⁻¹, and results are expressed as presence or absence.
 5 NaClO 1% solution is used as control.

Strain	Initial inoculum	Water rinsing	NaClO 1%	NaOH solution (pH 13.2)	NaOH solution (pH 13.2) + ozone (42.3 g Nm ⁻³)
<i>L. innocua</i>	7.5 ± 0.9	5.9 ± 0.3	< 0.3 Absence	< 0.3 Absence	< 0.3 Absence
<i>P. putida</i>	7.9 ± 1.0	6.5 ± 0.9	< 0.3 Absence	< 0.3 Absence	< 0.3 Absence

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1 **Table 2.** Evolution of the viability of *L. innocua* CECT 910 and *P. putida* DSM 12264
 2 in the cleaning wastewater after disinfection at 40°C and different times, 10, 20 and 30
 3 minutes. Initial inoculum is expressed as log CFU mL⁻¹. Experiments with pre-
 4 enrichment of culture medium were performed in cases where the detection limit was
 5 less than 0.3 log CFU mL⁻¹, and results are expressed as presence or absence. NaClO
 6 1% solution is used as control.

Test Strain	10 min		20 min		30 min	
	<i>L. innocua</i>	<i>P. putida</i>	<i>L. innocua</i>	<i>P. putida</i>	<i>L. innocua</i>	<i>P. putida</i>
Initial inoculum	5.58	2.12	5.82	4.23	5.82	5.08
NaClO 1%	Absence	Absence	Absence	Absence	Absence	Absence
NaOH solution (pH 13.2)	Absence	Absence	Absence	Absence	Absence	Absence
NaOH (pH 13.2) + ozone (42.3 g Nm ⁻³)	Absence	Absence	Absence	Absence	Absence	Absence

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