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Veterinary antibiotics and metals impact the mass, composition and hydrolysis of biomass cultivated in piggery wastewater treatment photobioreactors

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ABSTRACT

Photobioreactors are a promising alternative for piggery wastewater treatment which allows to produce valuable biomass, but the presence of antibiotics or heavy metals in the pig manure can influence the biomass composition and the posterior valorization processes. This study evaluates the effect of these pollutants on mass, composition and hydrolysis yields of the consortia of microalgae and bacteria obtained in a 1280 L photobioreactor treating pig manure. The photobioreactor feed was doped with 1) veterinary antibiotics; 2) copper, zinc, and arsenic; and 3) combination of both pollutants. The pollutants presence decreased the mass of biomass grown in the photobioreactor by up to 35% while glucose content of the biomass by up to 42% and increased protein and xylose by 30% and 16% due to oxidative stress. Likewise, they increased the protein solubilization yield by acid hydrolysis at 120 ºC by 32% while reduced glucose solubilization by 49% after alkaline hydrolysis at 120 ºC. Applying enzymatic hydrolysis and ultrasound assisted enzymatic extractions, glucose and xylose recoveries were drastically reduced in presence of heavy metals (~100%). Antibiotics increased xylose solubilization by ultrasonication (74%), but also its degradation, decreasing xylose recovery. Thus, the presence of all these pollutants in pig manure affected biomass production and valorization and these results must be considered for biorefinery processes.

1. Introduction

Pig manure has become a huge source of environmental pollution that must be treated to prevent contamination. This type of residue is rich in nutrients like organic matter, nitrogen, and phosphorus (Rojo et al., 2023) that must be eliminated prior to discharge. However, the concern about the presence of other microcontaminants as veterinary antibiotics and metals in the pig manure has increased in the last years (López-Serna et al., 2019) due to the severe environmental and health problems associated with them.

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Veterinary antibiotics (VA) are emerging pollutants used in farms to treat animal diseases and they are poorly absorbed by pig's digestive tract (Conde-Cid et al., 2020), hence most of them are excreted in the manure. Different types of antibiotics are found in pig manure, including sulphonamides (such as sulfadiazine), fluroquinolones (such as ciprofloxacin) and tetracyclines (López-Serna et al., 2019; Zambrano et al., 2023). Typical concentrations of these antibiotics in the liquid phase of the pig manure were below 1 mg/L (Van Epps and Blaney, 2016).

Metals (TE) are usually found in liquid pig manure because animal feed contains traces of these elements, including copper and zinc (Wang et al., 2023), which are essential micronutrients in animal growth. Arsenic can also be found because it is present in the well water. All these elements can be found with concentrations between 4.7 – 148 mg/L (copper), 12 – 234 mg/L (zinc), and <690 μg/L (arsenic) (Collao et al., 2022).

Both types of contaminants cannot be removed from piggery wastewater by conventional wastewater treatment plants (Amaro et al., 2023). Bioremediation with the microalgae-bacteria consortia present in wastewater treatment photobioreactors has emerged as a promising technology in terms of removal efficiency of contaminants thanks to the symbiosis between microalgae and bacteria and potential use of generated biomass to produce high-added value products (Singh et al., 2021; Upadhyay et al., 2021). Usually, VA and TE are removed by bio-adsorption and/or bioaccumulation where contaminants bind or are assimilated by microalgae (López-Pacheco et al., 2021; Rempel et al., 2021). So, after wastewater bioremediation it is possible that VA or TE would be present in the produced biomass and affect its macromolecular composition and characteristics (Leong and Chang, 2020).

As example, the carbohydrate content of *Chlorella* spp. grown on swine wastewater increased up to 52.7% from 40% while protein content decreased to 37% from 46% by addition of VA such as tetracycline (1 mg/L) and doxycycline (1 mg/L) (Michelon et al., 2022). On the other hand, TE favoured lipid accumulation, increasing its content in *Chlorella minutissima* with the addition of 0.4 mM of Cd^{2+} (21%) and 0.4 mM of Cu^{2+} (94%), but the protein content of *Chlorella vulgaris* decreased with the presence of cobalt (10^{-9} M), copper (10^{-9} M), and zinc (10^{-9} M) in a synthetic culture medium (Salama et al., 2019). However, there is very scarce research on the influence of these contaminants on the biomass composition and none on recovery processes after bioremediation treatment. Rempel et al. (2021b) investigated the effect of various emerging pollutants (paracetamol, diazepam, fluoxetine, acetylsalicylic acid, and caffeine) on chemical composition and carbohydrate extraction applying only enzymatic hydrolysis (with 1% v/v of Liquozyme Supre 2.2X and AMG 300 L) of various pure microalgae species (*Spirulina*, *Chlorella* and *Scenedesmus*) grown on synthetic media. Only acetylsalicylic acid and caffeine affected the carbohydrates and proteins content in all species, but there was not influence of any emerging pollutants in the carbohydrate extraction by enzymatic hydrolysis. Protein extraction was not investigated in this study. Regarding TE, it is demonstrated that they can inhibit the activity of some enzymes, which would consequently influence the performance of enzymatic hydrolysis. Among them, Zn^{2+} can inhibit many protease enzymes due its strong interactions with aspartic acid, glutamic acid, and cysteine (Maret, 2013) while oxidative metal ions can inhibit cellulase activity (Tejirian and Xu, 2010).

Currently, there is no research about the effect of these contaminants (VA and TE) on the enzymatic hydrolysis of biomass grown on wastewater, which is important to consider in the production of high-added value products in a valorization process. Besides enzymatic hydrolysis, there are other possible extraction methods which could be influenced by VA and TE, including chemical, physical, or assisted enzymatic methods that have provided interesting results with this microalgae-bacterial residual biomass (Rojo et al., 2023). Nevertheless, to the best of our knowledge, there is also no research about the impact of VA and TE in all these extraction methods nor in microalgae-bacteria biomass grown in wastewater. This research is pioneer in the study of the influence of three VA (sulfadiazine, tetracycline, and ciprofloxacin) and three TE (copper, zinc, and arsenic) in the composition and extraction by different methods of proteins and carbohydrates from microalgal-bacterial consortium grown on piggery wastewater. Composition and VA and TE content of the biomasses grown in no-doped and doped piggery wastewater were analyzed, along with the cellular structure by scanning electron microscopy (SEM). The extraction methods studied were chemical (acid and alkaline), enzymatic (with protease), ultrasounds, and ultrasound-assisted enzymatic hydrolysis. Protein and carbohydrate solubilization from biomasses were determined, along with peptide and monosaccharide recovery in the hydrolysates.

Table 1
Concentrations of veterinary antibiotics and heavy metals in each assay.

	Pollutant	Concentration
Assay 1 (VA)	Sulfadiazine (SDZ)	100 μg/L
	Tetracycline (TET)	100 μg/L
	Ciprofloxacin (CIP)	100 μg/L
Assay 2 (TE)	Copper ($CuCl_2 \cdot 2\ H_2O$)	20 mg/L
	Zinc ($ZnCl_2$)	20 mg/L
	Arsenic ($Na_2HAsO_4 \cdot 7\ H_2O$)	30 μg/L
Assay 3 (VA and TE)	Sulfadiazine (SDZ)	100 μg/L
	Tetracycline (TET)	100 μg/L
	Ciprofloxacin (CIP)	100 μg/L
	Copper ($CuCl_2 \cdot 2\ H_2O$)	20 mg/L
	Zinc ($ZnCl_2$)	20 mg/L
	Arsenic ($Na_2HAsO_4 \cdot 7\ H_2O$)	30 μg/L

2. Materials and methods

2.1. Biomass cultivation conditions

Three assays were carried out in a thin-layer cascade photobioreactor of 1280 L, inoculated with 1.1 g/L of Scenedesmus almeriensis CCAP 276/24 at 20% of their working volume and then filled up to the final 1280 L with pig slurry diluted at 10%. The photobioreactor operated with a dilution rate of 0.2 d^{-1} and biomass was harvested twice a day (biomass retention time = 5 days), fed with 10%-diluted piggery wastewater. The reactor had a culture depth of 0.02 m and a 1% slope that led to a culture velocity of 0.2 m/s and it was operated at a pH of 8 which was maintained by CO_2 injection. The water evaporation (approximate ~30%) was compensated every day (Ciardi et al., 2022). The proportion of bacteria in this type of biomass grown on piggery wastewater was estimated between 65% and 82% based on a biological model (Sánchez-zurano et al., 2021). Three antibiotics commonly used on pig farms and belonging to different types of drugs (sulfadiazine (SDZ), tetracycline (TET), and ciprofloxacin (CIP)) were selected for VA doping experiments and copper, zinc, and arsenic were added for TE doping experiments. The concentrations used in this study are shown in Table 1 and based on typical values found in pig manure (Collao et al., 2022; Van Epps and Blaney, 2016; Zambrano et al., 2023) and considering the feed dilution. Starting each assay, the photobioreactor was fed with no-doped piggery wastewater for 15 days until the steady state was reached to obtain no-doping biomasses (NB) as controls. Then, from day 16 to day 36, the feed was doped with three VA (SDZ, TET, CIP) in assay 1, three TE (copper, zinc, arsenic) in assay 2 and both types of pollutants (3 VA and 3 TE) in assay 3, to obtain the doping biomasses (DB). More days were planned to ensure steady state in the periods with doping, due to the stress on biomass growth caused by the presence of contaminants in the culture medium. After each assay, the photobioreactor was emptied and cleaned before starting the next experiment. Assay 1 was carried out in November with an average solar radiation of 11 MJ/m^2 (range between 9 and 13.2 MJ/m^2) and average daily temperatures ranging from 15 to 20 ºC. Assay 2 was performed in March with an average solar radiation of 18 MJ/m^2 (range between 15.7 and 22.2 MJ/m^2) and average daily temperatures ranging from 15 to 16 ºC. Finally, assay 3 was carried out in May with an average solar radiation of 26 MJ/m^2 (range between 21.4 and 28.4 MJ/m^2) and average daily temperatures ranging from 18 to 23 ºC. The biomass concentration of the photobioreactor was determined every day as total suspended solids (TSS), and the concentration of total nitrogen (TN), total phosphorus (TP), and total organic content (TOC) in the feed and effluent of the photobioreactor.

Biomass samples were harvested at the end of each period (in the days 15 and 36 respectively), centrifugated, and freeze-dried to obtain the NB and DB samples. These samples were analyzed to obtain its composition (protein, amino acid profile, carbohydrates, and lipid) and were subjected to the hydrolysis methods described below (Section 2.2) to study the influence of the pollutants in the extraction method. Likewise, the obtained biomass of assay 1 was analyzed for the VA content, the biomass of assay 2 was analyzed for the TE content and the biomass of assay 3 was analyzed for the VA and TE content.

2.2. Hydrolysis methods

Several hydrolysis methods were carried out to extract proteins and carbohydrates from the different biomasses collected from the photobioreactor according to (Rojo et al., 2023), which operation conditions were described in the Table 2. In brief, chemical methods at 120 ºC (NaOH 120 and HCl 120) were carried out in an autoclave at pressure of 1 bar using 2 M NaOH and HCl respectively. These methods allow to achieve high solubilization yields from microalgae biomasses grown on piggery wastewater of up to 90% of carbohydrates with acid hydrolysis and >80% of proteins with alkaline hydrolysis (Rojo et al., 2023). On the other hand, physical methods with ultrasounds (UAE and UAEE-P) were performed with an ultrasonic probe UIP1000hd (Hielscher Ultrasound Technology, Germany) at 50% of amplitude in a temperature-controlled jacketed vessel at 50 ºC and pH of 6.5. Ultrasonication is considered an efficient technology which can achieve high extraction yields in short times without affecting the molecules properties due to the mild operation conditions (Zheng et al., 2021). Finally, enzymatic methods (UAEE-P and HE-P) were performed using Protamex as enzyme with a concentration of 1:100 $w/w_{dry\ biomass}$. This enzyme is an endo-protease that can solubilize proteins selectively providing peptides with promising sizes (Rojo et al., 2021), while the application of ultrasound in combination with enzymes can improve the extraction efficiency of enzymatic hydrolysis with short hydrolysis time (Rojo et al., 2023). All the hydrolysis experiments were performed for 1 hour with a biomass concentration of 5% ($w_{dry\ biomass}/w$) and working volumes of 250 mL.

Fig. 1 shows the experimental method of the hydrolysis experiments. After hydrolysis experiments, the biomass suspensions were centrifuged at 7800 rpm for 10 min to separate the solid waste (solid fraction) and the hydrolysate (liquid fraction). The solid fractions were freeze-dried for the following analysis. Weights, total and volatile solid, and nitrogen concentrations were determined in both

Table 2
Operation conditions of the different extraction methods.

Extraction method	T (ºC)	pH	Enzyme/ Reagent	Concentration
Ultrasonic assisted extraction (UAE)	50	6.5	-	-
Ultrasonic assisted enzymatic extraction (UAEE - P)	50	6.5	Protamex	1:100 w/w
Enzymatic hydrolysis (HE – P)	50	6.5	Protamex	1:100 w/w
Alkaline hydrolysis (NaOH 120)	120	-	NaOH	2 M
Acid hydrolysis (HCl 120)	120	-	HCl	2 M

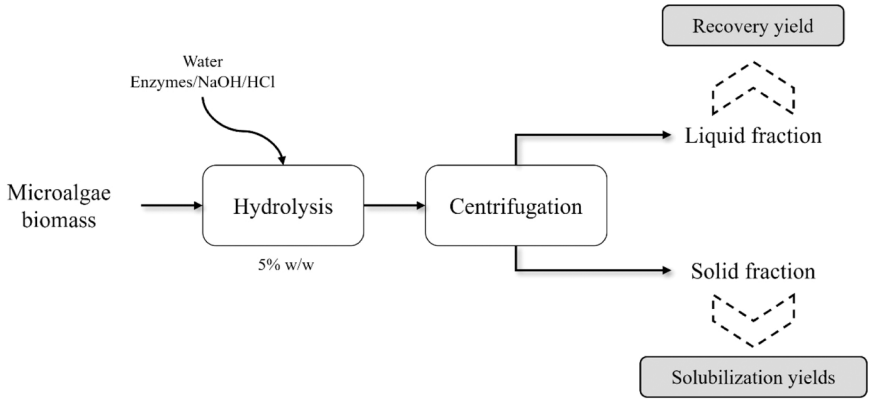

Fig. 1. Diagram of the hydrolysis method.

fractions to check mass balances. Protein, carbohydrate, VA, and TE content were determined in the solid residual fractions, while peptide, and monosaccharide concentrations were analyzed in the hydrolysates. Finally, the initial biomasses of all assays were analyzed by scanning electron microscopy (SEM) to analyze the changes in cellular structure by the presence of VA and TE.

2.3. Analytical methods

Total suspended solids (TSS), total solid (TS), and volatile solid content (VS) were determined by a gravimetric method (Rojo et al., 2023). Total nitrogen (TN) and total phosphorus (TP) were analyzed in the feed and effluent of the photobioreactor by Total Nitrogen Kjeldahl method and colorimetry based on standard methods, while TOC was determined using a TOC-V CSH analyzer equipped with a TNM-1 chemiluminescence module (Shimadzu, Kyoto, Japan). Protein content in the initial biomasses and both fractions after hydrolysis experiments was determined using the Total Nitrogen Kjeldahl method and applying a nitrogen-protein factor obtained from the amino acid profile of each used biomass according to (Rojo et al., 2021). The total amino acid profile of the initial biomasses was analyzed by HPLC according to internal analytical protocol of the Instrumental Techniques Laboratory (LTI – UVa) described in (Rojo et al., 2021). Carbohydrate content in the initial biomasses and solid waste fractions was determined as monosaccharides (glucose, xylose, cellobiose, and arabinose) after a concentrated acid hydrolysis with H_2SO_4, based on a NREL procedure (Martin Juárez et al., 2021). The monosaccharide concentrations in the liquid fractions were quantified by high-performance liquid chromatography (HPLC) using a Shimadzu LC-2050 (Japan), a refractive index detector RID-20A (Japan) with a Bio-Rad HPX-87 H ion-exclusion column and external standards. Lipid content in the initial biomasses was determined using a modified protocol based on a chloroform-methanol 2:1 extraction (Lee et al., 2020). The VA biomass content was analyzed by ultra-high performance liquid chromatography (UHPLC) coupled with mass spectrometry (MS/MS) according to the method described in (López-Serna et al., 2019). Copper (Cu) and zinc (Zn) biomass content was analyzed by inductively coupled plasma spectrometry coupled with an optical emission spectrophotometer (ICP-OES) while arsenic (As) content was analyzed by inductively coupled plasma source mass spectrometer (ICP-MS) according to (Collao et al., 2022). For these TE analysis, initial biomass and solid fractions were previously hydrolyzed with nitric acid (HNO_3) at 0.1 M. Finally, electronic micrographs were taken using a Jeol JSM-820 scanning electronic microscope (SEM).

2.4. Calculations

TN, TP, TOC, VA and TE removals in the photobioreactor were determined with Eq. 1 (Collao et al., 2022):

$$Removal \quad (\%) = \frac{(C_{input} \cdot Q_{input} - C_{output} \cdot Q_{output})}{C_{input} \cdot Q_{input}} \times 100 \tag{1}$$

where C_{input} and C_{output} represent the concentrations of TN, TP, TOC, VA, and TE in the feed and liquid phase of the effluent of the photobioreactor, respectively. Q_{input} and Q_{output} correspond to the feed and effluent flows of the photobioreactor.

Solubilization yields of the main biomass compounds (proteins and carbohydrates) were calculated with Eq. 2:

$$Compound \quad solubilization \quad \left(\frac{g \quad compound}{100g \quad biomass}\right) = \left(\frac{M_{initial \quad biomass} \cdot C_{initial \quad biomass} - M_{solid \quad waste} \cdot C_{solid \quad waste}}{M_{initial \quad biomass}}\right) \tag{2}$$

where $M_{initial\ biomass}$ was the mass of the initial biomass (g), $C_{initial\ biomass}$ was the compound mass content in the initial biomass (%), $M_{solid\ waste}$ was the mass of the solid residue after hydrolysis (g), and $C_{solid\ waste}$ was the compound mass content in the solid residue after hydrolysis (%). On the other hand, compound recovery as peptides and monosaccharides were calculated with Eq. 3:

$$Compound \quad recovery \quad \left(\frac{g \quad compound}{100g \quad biomass}\right) = \frac{M_{hydrolysate} \cdot C_{hydrolysate}}{M_{initial \quad biomass}} \tag{3}$$

where $M_{hydrolysate}$ was the mass of the hydrolysate after hydrolysis (g), and $C_{hydrolysate}$ was the compound mass content in the hydrolysate after hydrolysis (%). Solubilization and recovery yields (%) were determined with Eqs. 4 and 5:

$$Compound\quad solubilization\quad yield\quad (\%) = \left(1 - \frac{M_{solid\quad waste} \cdot C_{solid\quad waste}}{M_{initial\quad biomass} \cdot C_{initial\quad biomass}}\right) \times 100 \tag{4}$$

$$Compound\quad recovery\quad yield\quad (\%) = \left(\frac{M_{hydrolysate} \cdot C_{hydrolysate}}{M_{initial\quad biomass} \cdot C_{initial\quad biomass}}\right) \times 100 \tag{5}$$

2.5. Statistical analysis

The differences among the mean compositions and yields were analyzed by the least significant difference test (LSD) at a confidence level of 95% ($\alpha = 0.05$) using the software Statgraphics Centurion 19. All the analysis were performed by duplicate and the results were expressed as the mean ± standard deviation.

3. Results and discussion

3.1. Piggery wastewater treatment performance

Biomass concentration in the photobioreactor during no-doping phase was 0.75 g/L in assay 1 (November and solar radiation of ~9 MJ/m^2), 1.85 g/L in assay 2 (March and solar radiation of ~15.7 MJ/m^2) and 2.42 g/L in assay 3 (May and solar radiation of ~21.4 MJ/m^2). These results evidenced the positive effect of solar radiation in biomass productivity. Thus, it can be observed that the period of the year in which the treatment is performed is also an important operational factor.

The biomass concentration in the photobioreactor decreased in the doping phase in assays 2 and 3, being 0.75, 1.10, and 1.19 g/L in assays 1, 2 and 3 respectively. The presence of TE decreased the biomass productivity despite the slight increase in solar radiation in the doping phase in assays 2 and 3 that favors biomass productivity (Rojo et al., 2022). Therefore, the decrease in biomass concentration observed in assays 2 and 3 in the second period (during doping) could be attributed to oxidative stress of the TE (Leong and Chang, 2020). In agreement with the results of biomass productivity, no significant effect of VA on the removal of TN, TP, and TOC was found in assay 1. However, the doping with TE in assays 2 and 3 decreased the removals of organic matter and nutrients, from values around 80–70% for TOC, 93–70% for TN and 89–68% for TP. This effect of metals was also reported by (Li et al., 2020) who found a decrease in TP and nitrogen removal in presence of Zn^{2+} from swine wastewater attributed to growth inhibition.

None of the analyzed VA and TE were detected in the control no-doping biomasses (NB) of the three assays. In the Supplementary material were defined the material balances of these VA and TE in the photobioreactor. In assay 1, after doping the photobioreactor feed with the three VA, the obtained doping biomass (DB) achieved CIP, TET and SDZ concentrations of 74, 79, and 76 μg/g respectively by removing more than 80% of VA from the photobioreactor feed, which are similar results to those previously reported by (Zambrano et al., 2021). On the other hand, in assay 2 the DB contained 20 mg/g of Cu, 16 mg/g of Zn, and 20 μg/g of As. The TE removal efficiency in the photobioreactor was >80% for Cu and Zn, and 96% for As, similar values reported by (Collao et al., 2022) working with CSTR open photobioreactors fed with piggery wastewater diluted at 5% v/v. Finally, in assay 3 the CIP, TET and SDZ content in the DB was only 18, 1, and 2 μg/g respectively by removing 68% of SDZ from the photobioreactor feed. CIP and TET were not detected in the photobioreactor output, and together with the low concentration of VA in the biomass in comparison with assay 1,

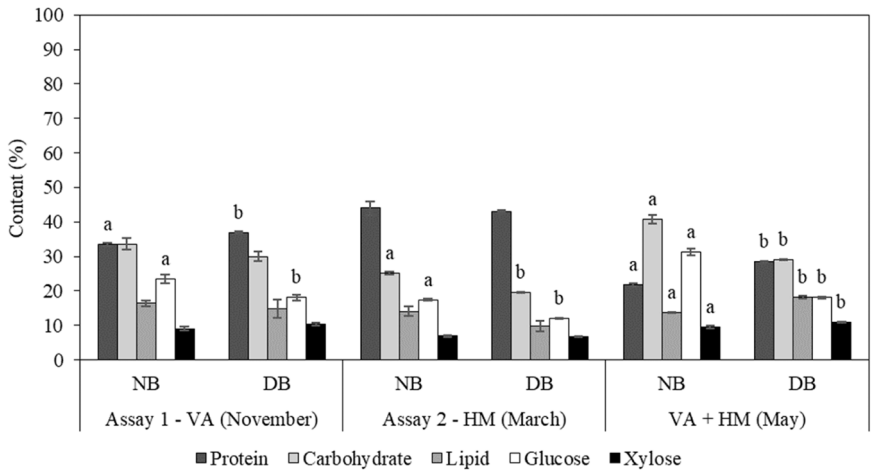

Fig. 2. Biomass composition (ash-free dry basis). The data are provided as means ± standard deviations of 2 analytical determinations. Mean values with different letters are significantly different for $\alpha < 0.05$ by the LSD Test.

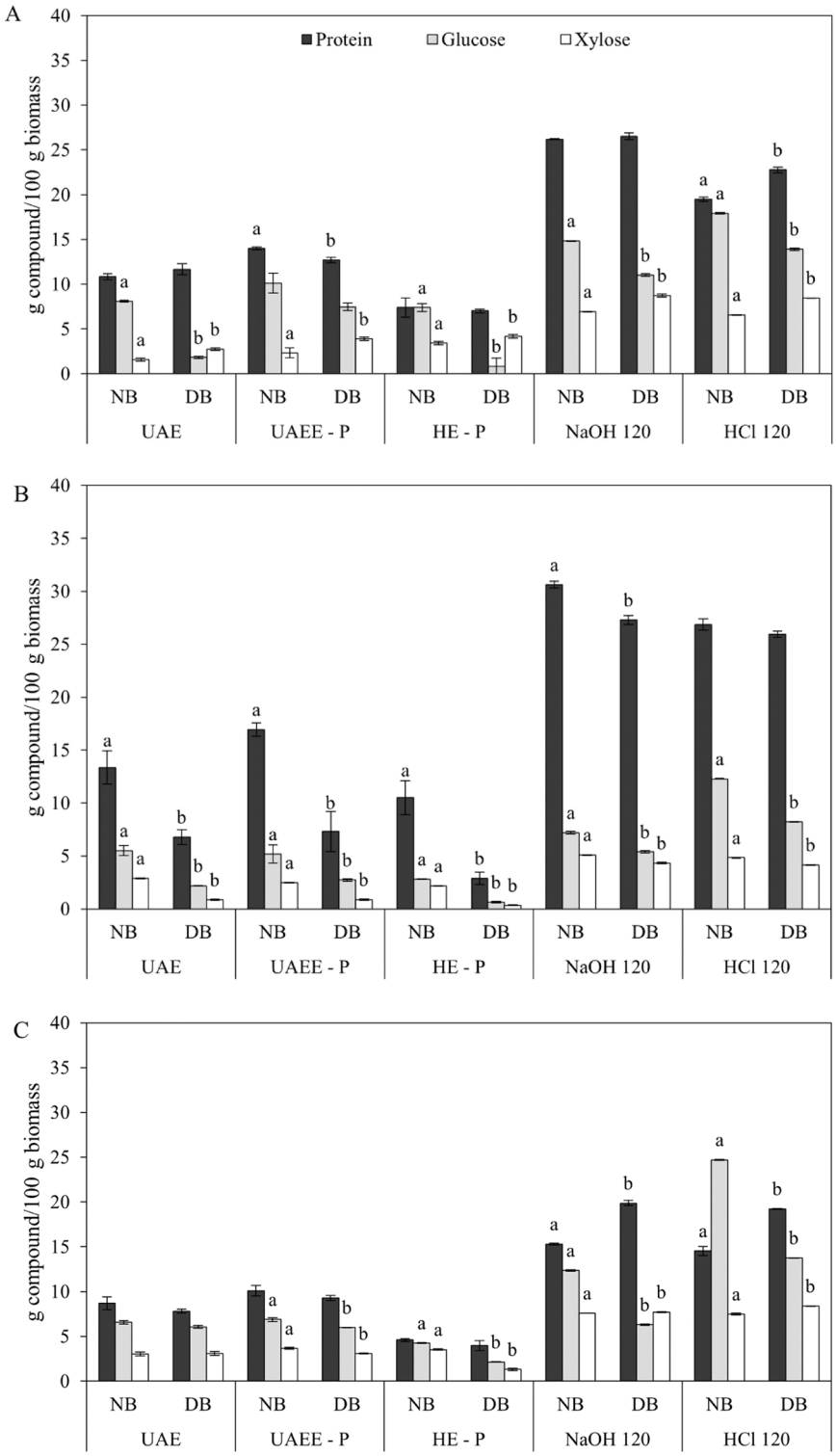

Fig. 3. Protein solubilization ($g_{proteins}$ /100 $g_{biomass}$), glucose solubilization ($g_{glucose}$ /100 $g_{biomass}$) and xylose solubilization (g_{xylose} /100 $g_{biomass}$) in reference to the initial microalgal biomass in the assay 1 with VA (A), assay 2 with TE (B) and assay 3 with VA and TE (C). The data are provided as means ± standard deviations of 2 analytical determinations and the standard deviation of the means is represented by vertical interval lines. Different letters denote remarkable differences ($\alpha < 0.05$) according to LSD test.

could indicate a higher degradation of these compounds in presence of TE. Finally, Cu, Zn and, As content was 15, 14 mg/g, and 21 μg/g with removal efficiencies in the photobioreactor higher than 85% for the three TE. So, after bioremediation, all the doped contaminants were present in the biomass and thus, the final application of the biomass and the extracted compounds are limited by the concentration of these pollutants on the final products (Rojo et al., 2022). It is necessary to consider that this type of biomass grown on piggery wastewater cannot be used in pharmacy or in human consumption applications. Also, the influence of the downstream processes of this biomass should be studied to determine the effect in the final concentration of the pollutants in the products.

3.2. Effect of VA and TE on biomass composition

Fig. 2 shows the compositions (proteins, carbohydrates, lipids) of freeze-dried biomasses obtained after wastewater microalgae treatment in each assay before and after doping. The different biomass compositions in different assays are related to the time of the year in which each experiment was carried out. High protein and low carbohydrate contents are expected during winter operation, while high carbohydrate and low protein contents are obtained during spring operation (Martin Juárez et al., 2021).

VA presence in the DB had a slight influence on the microalgae biomass composition of the assay 1, with significant differences between no-doping and doping biomass in protein and glucose content ($\alpha < 0.05$). Protein content increased from 33.6% to 37% while glucose content decreased from 23.6% to 18.1%. These changes produced by the presence of VA in the composition of the biomass could be attributed to the oxidative stress caused by the imbalance in the reactive oxygen species (ROS) (Rempel et al., 2022; Wang et al., 2022). (Chen et al., 2020) showed that in presence of 270 mg/L SDZ, protein content of *Chlorella vulgaris* grown on synthetic media with BG-11 increased by 168% while carbohydrate content slightly decreased in presence of 30 mg/L of SDZ. Probably, the low VA concentration of our study (only 100 μg/L) compared to those used by (Chen et al., 2020) resulted in lower change in protein content, although the same behavior to overcome the oxidative stress was observed.

Regarding assay 2, the microalgae composition was also altered by the presence of TE in the DB, obtaining significant differences between no-doping and doping biomass ($\alpha < 0.05$) in glucose percentages (and thus, carbohydrates) which decreased from 17.4% to 12.2%. This reduction has been again attributed to oxidative stress and formation of ROS which cause alterations of microalgae biological characteristics and damages in cell wall compounds and structure by metals (Danouche et al., 2022). Zn could bind to thiols and led to intracellular metal accumulation and production of ROS that damaged carbohydrates (Birben et al., 2012; Zhou et al., 2018), while Cu could inhibit glucose biosynthesis (Aggarwal et al., 2011).

In assay 3, the combined effect of VA and TE significantly changed all biomass macrocompounds content (proteins, carbohydrates, and lipids) as indicated by the LSD test ($\alpha < 0.05$). In this case, the protein, xylose, and lipid contents did increase by 30%, 16% and 33% respectively while glucose content decreased by 42%, resulting in 29% lower carbohydrate content. Therefore, the presence of both types of pollutants had resulted in a big oxidative stress, causing an increase in protein synthesis to overcome the imbalance due to overproduction of ROS (Rempel et al., 2022; Wang et al., 2022) at the expense of the glucose content. Xylose shows the same behavior as proteins, associated with glycoproteins (Rojo et al., 2021). The lipid content also increased, but its low concentration compared to the rest of components, make it not worth studying.

The presence of the two types of contaminants also modified cell structure and morphology, as shown by scanning electron microscopy (SEM) of the different doping biomasses. In assay 1, the presence of VA slightly increased the roughness of the cell wall in DB compared to NB. (Zambrano et al., 2021) studied the removal of different VA (tetracycline, ciprofloxacin, and sulfadiazine) by microalgae-bacteria biomass and found a clear and similar alteration of the cell surface roughness. These changes were attributed to the binding of VA to different functional groups of the cell wall which altered the cellular structure. Likewise, the presence of TE also varied the morphology of the biomass with rougher cell surface, attachment of cells by filaments and the presence of crystallized salts in the DB compared to NB. The same changes were observed by (Urrutia et al., 2019), who studied the effect of heavy metals (Cu and Mo) on *Chorella vulgaris* morphology and found changes and damages in the morphology. All these changes observed in the SEM images were a defense mechanism against antibiotic and metal toxicity to preserve algae cells from serious damage produced by these contaminants and protect internal content (Gomaa et al., 2021).

3.3. Effect of VA on protein and carbohydrate solubilization

Several types of hydrolysis were applied to the biomasses of assay 1 for the extraction of proteins and carbohydrates. All mass balances of nitrogen and volatile solids were determined, and very low losses were obtained, being always less than 7.7% and 4.2% respectively in all experiments. The amount of N supplied by the protease enzyme was considered negligible for all calculations in enzymatic treatments, since the enzyme/substrate ratio in the experiments was very low (Rojo et al., 2023).

Fig. 3.A shows the amounts of proteins and carbohydrates solubilized from NB and DB after each hydrolysis treatment in assay 1. As expected from previously published works (Martin Juárez et al., 2021; Rojo et al., 2023), chemical hydrolysis with NaOH at 120 °C provided the highest protein solubilization and acid hydrolysis with HCl at 120 °C the highest carbohydrate solubilization. Previous studies (Martín-Juárez et al., 2019) showed that the presence of bacteria in the microalgal biomass significantly influences protein and carbohydrate solubilization by applying chemical hydrolysis. Specifically, alkaline hydrolysis at 120 °C provided higher carbohydrate (58%) and protein (70%) solubilization yields from microalgae grown in synthetic medium without bacteria than from biomass grown on piggery wastewater (50% and 67% respectively). The presence of bacteria in the biomass grown on piggery wastewater resulted in higher carbohydrate solubilization by acid hydrolysis at 120 °C (70%) compared to pure microalgae grown on synthetic medium (62%).

The presence of VA did not significantly affect the protein solubilization by alkaline hydrolysis at 120 °C, achieving 26.2 $g_{proteins}$

/100 $g_{biomass}$ from NB and 26.5 $g_{proteins}$ /100 $g_{biomass}$ from DB. but the difference in carbohydrate solubilization was significant ($\alpha < 0.05$). The doping of VA decreased glucose solubilization by alkaline hydrolysis from 14.9 $g_{glucose}$ /100 $g_{biomass}$ (NB) to 11 $g_{glucose}$ /100 $g_{biomass}$ (DB), while xylose solubilization increased from 6.9 g_{xylose} /100 $g_{biomass}$ (NB) to 8.7 g_{xylose} /100 $g_{biomass}$ (DB). Significant differences ($\alpha < 0.05$) were also found in terms of carbohydrate solubilization yields (decreasing from 69.9% to 66.6% for glucose but increasing from 86% to 92.2% for xylose), indicating possible structural changes in the cell that affected the extraction. Should be taken into consideration that alkaline hydrolysis is usually the most efficient method for extracting proteins (Rojo et al., 2023), and this high efficiency could override the VA effect.

On the other hand, chemical hydrolysis with HCl at 120 ºC achieved sobulizations of 24.5 $g_{carbohydrates}$ /100 $g_{biomass}$ from NB and 22.4 $g_{carbohydrates}$ /100 $g_{biomass}$ from DB with significant differences ($\alpha < 0.05$). As occurred in the alkaline hydrolysis, glucose solubilization decreased in presence of VA (17.9 $g_{glucose}$ /100 $g_{biomass}$ from NB and 13.9 $g_{glucose}$ /100 $g_{biomass}$ from DB) while xylose solubilization increased (6.6 g_{xylose} /100 $g_{biomass}$ from NB and 8.5 g_{xylose} /100 $g_{biomass}$ from DB) with significant differences in both cases ($\alpha < 0.05$). The LSD test also found significant differences in xylose solubilization yields by acid hydrolysis, increasing from 81.4% with NB to 89.3% with DB. Some VA, like tetracyclines or sulfonamides are very sensitive to pH and at alkaline conditions, since tetracyclines suffer an epimerization transforming into other compounds (Michelon et al., 2022) but acid conditions result in high degradation of these VA and other toxic organic compounds as 4-epianhydrotetracycline can be formed (Roy, 2011), which could explain why acid hydrolysis was most affected by the VA. (Roy, 2011)

Regarding physical and biological methods, lower protein, and carbohydrate solubilization yields were obtained than by chemical treatments. (Rojo et al., 2021) investigated the effect of the presence of bacteria on enzymatic hydrolysis of microalgae biomass working with different enzymes. They observed that bacteria favored carbohydrate solubilization from biomass grown on piggery wastewater (up to 38.5% with cellulase enzyme and 5 h of hydrolysis), while protein solubilization was higher from pure microalgae grown on synthetic medium (up to 64.8% with protease enzyme and 5 h of hydrolysis).

According to the LSD test, the presence of VA only significantly influenced ($\alpha < 0.05$) the protein solubilization by UAEE-P, reducing the amount solubilized from 14 $g_{proteins}$ /100 $g_{biomass}$ to 12.7 $g_{proteins}$ /100 $g_{biomass}$. Glucose solubilization decreased in presence of VA with significant differences ($\alpha < 0.05$) after applying UAE (from 8.1 $g_{glucose}$ /100 $g_{biomass}$ to 1.8 $g_{glucose}$ /100 $g_{biomass}$) and HE-P (from 7.4 $g_{glucose}$ /100 $g_{biomass}$ to 0.9 $g_{glucose}$ /100 $g_{biomass}$) while the combination of ultrasounds and enzymes (UAEE-P) was able to extract this component (7.5 $g_{glucose}$ /100 $g_{biomass}$) without significant differences between NB and BD. The adsorption of SDZ and TET by the polysaccharides of the microorganism cells (Zambrano et al., 2021) could affect the macromolecular bindings, reducing the solubilization of glucose by mild methods. However, the combination of ultrasounds and enzymes was able to break down the VA binds, allowing glucose solubilization. Significant differences were also detected ($\alpha < 0.05$) for xylose solubilization in all these physical and biological methods, slightly increasing this monosaccharide solubilization with VA presence.

3.4. Effect of TE on protein and carbohydrate solubilization

As in the previous assay 1, all mass balances of volatile solids and nitrogen were determined in the experiments carried out in this assay 2 with TE. Low losses of nitrogen and volatile solids were obtained, being always lower than 7.2% and 6.5%.

Fig. 3.B shows the solubilized amounts of proteins and carbohydrates by the different hydrolysis treatments for microalgal biomasses before (NB) and after (DB) doping of pig manure in assay 2. Again, chemical hydrolysis with NaOH at 120 ºC provided the best results for protein solubilization, achieving 30.6 $g_{proteins}$ /100 $g_{biomass}$ from NB and 27.3 $g_{proteins}$ /100 $g_{biomass}$ from DB (co-solubilizing 7.2 $g_{glucose}$ /100 $g_{biomass}$ and 5.1 g_{xylose} /100 $g_{biomass}$ from NB and 5.4 $g_{glucose}$ /100 $g_{biomass}$ and 4.3 g_{xylose} /100 $g_{biomass}$ from DB). Thus, the presence of TE did influence protein and carbohydrate extraction by the alkaline hydrolysis at 120 ºC as indicated by the LSD test ($\alpha < 0.05$). However, the same statistical analysis did not find significant differences in protein (~87%) and xylose (~90%) solubilization yields, while glucose solubilization yield increased with TE doping from 51.3% from NB up to 62.1% from DB, with significant differences ($\alpha < 0.05$). Therefore, the variations in protein solubilization could be due to the slight differences in compositions between biomasses before and after doping. TE also induces the production of extracellular polymeric substances (EPS) which alters the cellular structure (Naveed et al., 2019). So, these changes possibly increase the availability of glucose, partly offsetting its lower concentration in the biomass.

On the other hand, chemical hydrolysis with HCl at 120 ºC provided moderate carbohydrate solubilization with significant differences between NB and DB but solubilized similar amounts of proteins from both biomasses (26 $g_{proteins}$ /100 $g_{biomass}$). As well as in the alkaline hydrolysis, the presence of TE decreased the amounts solubilized of glucose (from 12.3 $g_{glucose}$ /100 $g_{biomass}$ to 8.2 $g_{glucose}$ /100 $g_{biomass}$) and xylose (from 4.8 $g_{glucose}$ /100 $g_{biomass}$ to 4.2 $g_{glucose}$ /100 $g_{biomass}$). In terms of solubilization yields after acid hydrolysis, significant differences were observed only for glucose, increasing from 87.3% (NB) to 94.7% (DB). HCl has a high ability to desorb metals from biomass by damaging the metal-binding sites such as polysaccharides on the cell membrane surface (Manikandan et al., 2022). Again, the lower initial concentration of carbohydrates in the doping biomass (25.1% in NB vs 19.7% in DB) counteracts the increase on glucose solubilization yield by the presence of TE, resulting in an overall lower amount of solubilized carbohydrates from doping biomass.

Regarding physical and biological treatments, the negative influence of TE on the extraction process of proteins and carbohydrates from the DB was very evident in all the experiments (confirmed by the LSD test with $\alpha < 0.05$). The amount of solubilized proteins decreased by the presence of TE after applying UAE (from 13.3 $g_{proteins}$ /100 $g_{biomass}$ to 6.8 $g_{proteins}$ /100 $g_{biomass}$), UAEE-P (from 16.9 $g_{proteins}$ /100 $g_{biomass}$ to 7.3 $g_{proteins}$ /100 $g_{biomass}$), and HE-P (from 10.5 $g_{proteins}$ /100 $g_{biomass}$ to 2.9 $g_{proteins}$ /100 $g_{biomass}$). The same behavior was observed in the solubilization of carbohydrates (glucose and xylose), decreasing from 8.4 $g_{carbohydrates}$ /100 $g_{biomass}$ to 3 $g_{carbohydrates}$ /100 $g_{biomass}$ in the UAE, from 7.7 $g_{carbohydrates}$ /100 $g_{biomass}$ to 3.6 $g_{carbohydrates}$ /100 $g_{biomass}$ in the UAEE-P, and from

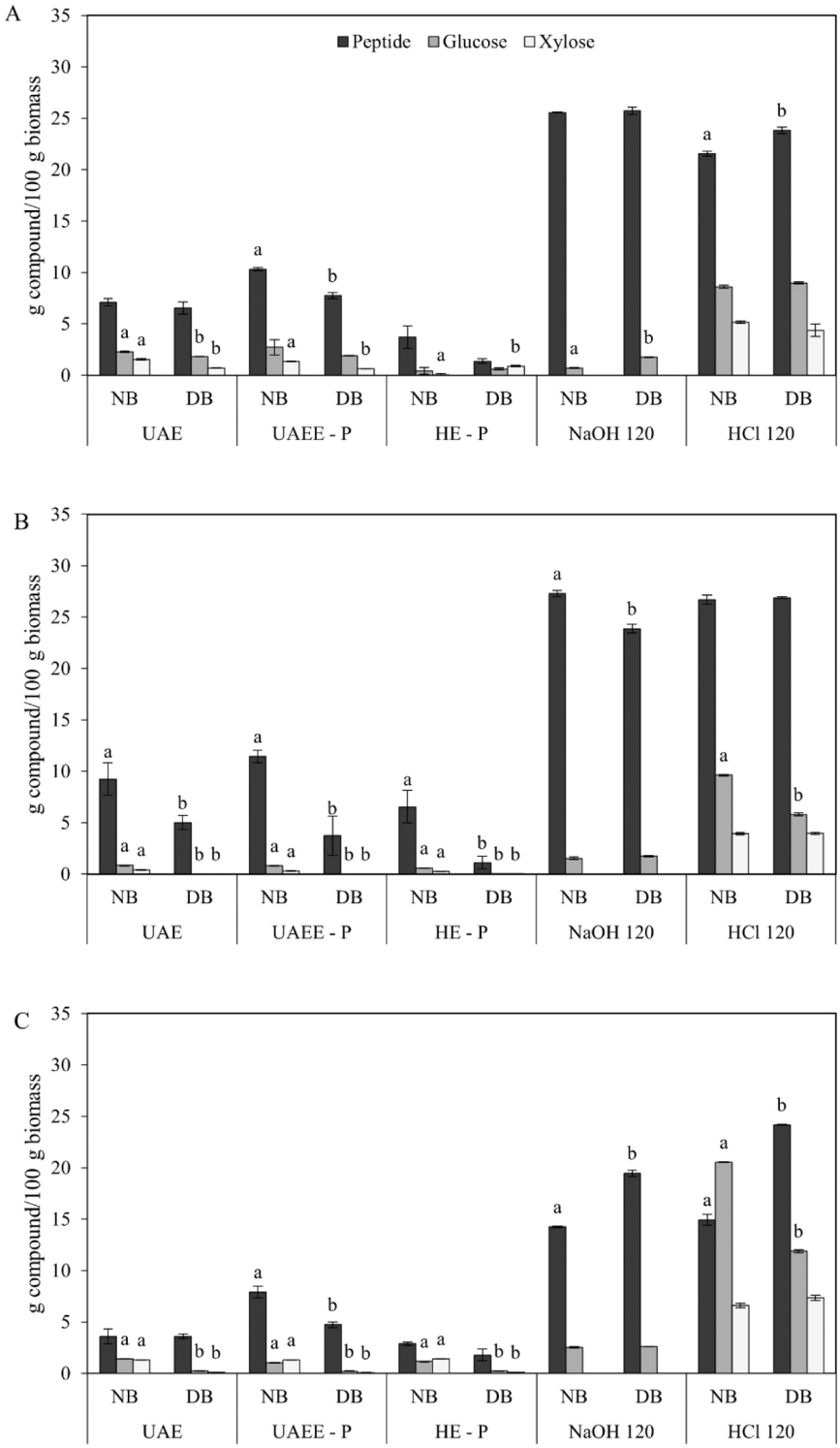

Fig. 4. Peptide, glucose, and xylose recovery yield ($g_{compound}$ /100 $g_{biomass}$) in reference to the initial microalgal biomass in the assay 1 with VA (A), assay 2 with TE (B) and assay 3 with VA and TE (C). The data are provided as means ± standard deviations of 2 analytical determinations and the standard deviation of the means is represented by vertical interval lines. Different letters denote remarkable differences ($\alpha < 0.05$) according to LSD test.

5 $g_{carbohydrates}$ /100 $g_{biomass}$ to 1 $g_{carbohydrates}$ /100 $g_{biomass}$ in the HE-P. In all cases, significant differences were also found in protein and carbohydrate solubilization yields ($\alpha < 0.05$), with TE reducing all solubilization yields. The decrease in yields of enzymatic treatments by the presence of TE was expected because enzymes are usually inhibited by the presence of heavy metals, among them zinc (Maret, 2013) and arsenic (Finnegan and Chen, 2012), which can bind to the thiol groups of protein enzymes, reducing the enzymatic activity. Also, the ions compete for biding sites interfering the enzymatic activity (Smith et al., 2022). The same negative effect was observed by (Tejirian and Xu, 2010), who studied the enzymatic cellulose hydrolysis of pretreated corn stover (lignocellulosic biomass), obtaining also remarkable decrease on hydrolysis yields by the presence of some metal's ions (including Cu^{2+} and Zn^{2+}).

3.5. Effect of VA and TE on protein and carbohydrate solubilization

All mass balances of volatile solids and nitrogen were determined in the experiments carried out in assay 3 with both types of pollutants. Low losses of nitrogen and volatile solids were obtained, being always less than 9.8% and 8.7%.

Regarding both chemical treatments at 120 ºC, significant differences were observed with the LSD test in the solubilization of proteins and carbohydrates ($\alpha < 0.05$) as shown in Fig. 3.C. The amounts of proteins solubilized with both treatments (acid and alkaline) were lower from NB (15.3 $g_{proteins}$ /100 $g_{biomass}$ and 14.5 $g_{proteins}$ /100 $g_{biomass}$) than from DB (19.9 $g_{proteins}$ /100 $g_{biomass}$ and 19.2 $g_{proteins}$ /100 $g_{biomass}$). On the contrary, the glucose solubilization decreased significantly ($\alpha < 0.05$) from 12.4 $g_{glucose}$ /100 $g_{biomass}$ (NB) to 6.3 $g_{glucose}$ /100 $g_{biomass}$ (DB) for alkaline treatment and from 24.7 $g_{glucose}$ /100 $g_{biomass}$ (NB) to 13.8 $g_{glucose}$ /100 $g_{biomass}$ (DB) for acid treatment. No significant effect of doping on the amounts of xylose solubilized was found for alkaline treatment, while for acid hydrolysis, solubilization increased from 7.5 g_{xylose} /100 $g_{biomass}$ (NB) to 8.4 g_{xylose} /100 $g_{biomass}$ (DB). In terms of solubilization yields, significant differences were also found ($\alpha < 0.05$), but increasing with doping in all the cases, although only slightly for carbohydrates. The highest protein solubilization yield was obtained after alkaline hydrolysis from DB (82.6%) and the highest carbohydrate solubilization yield was obtained after acid hydrolysis from DB (90%). This behavior may be related to a high oxidative stress in presence of both types of pollutants (Wang et al., 2022) which could reduce the membrane resistance to chemical treatment more than in the assays 1 and 2 for carbohydrate extraction. In the case of the proteins, to this increase in yield must be added their higher concentration in the doping biomass (Fig. 1).

For physical and biological treatments, the amounts of solubilized proteins were not influenced by the presence of combined contaminants. Significant differences ($\alpha < 0.05$) were found in carbohydrate solubilization after UAEE-P and HE-P treatments, decreasing the amount of solubilized glucose and xylose in presence of the pollutants (from 10.6 $g_{carbohydrates}$ /100 $g_{biomass}$ to 9.1 $g_{carbohydrates}$ /100 $g_{biomass}$ and from 7.8 $g_{carbohydrates}$ /100 $g_{biomass}$ to 3.5 $g_{carbohydrates}$ /100 $g_{biomass}$ respectively). Comparing the glucose solubilization yields, the only treatment affected by doping was UAEE-P, with an increase from 22.4% to 39.2%. The xylose solubilization yields decreased significantly after UAEE-P (from 40.6% to 33.2%) and after HE-P (from 39.7% to 13.6%).

3.6. Effect of VA on peptide and monosaccharide recovery

After solubilization of the macro compounds, not all of them can be recovered since losses and degradation occur during the hydrolysis process. Also, few research studies determine the real recovery of these compounds in the hydrolyzate after degradation of solubilized compounds. (Martín-Juárez et al., 2019) found that the presence of bacteria in the microalgal biomass exerted a negative influence on monosaccharide recovery after applying chemical hydrolysis (acid and alkaline), attributed to the increase on losses by microbial degradation. (Rojo et al., 2021) also studied the effect of the presence of bacteria on the recovery of peptides and monosaccharides by enzymatic hydrolysis, but in this case the losses resulted higher for pure microalgae grown on synthetic media (~20%) than for biomass grown on piggery wastewater (~9%).

Fig. 4.A shows the recovered amounts of peptides, glucose, and xylose from the biomass of assay 1 with VA. Chemical treatments at 120 ºC achieved the highest recovery results (as for solubilization yields), highlighting the high peptide recoveries after alkaline treatment (~25 $g_{peptides}$ /100 $g_{biomass}$ from NB and DB) and the high peptide (21.5 $g_{peptides}$ /100 $g_{biomass}$ from NB and 23.8 $g_{peptides}$ /100 $g_{biomass}$ from DB) and moderate glucose (~9 $g_{glucose}$ /100 $g_{biomass}$) recoveries after acid treatment. Likewise, xylose was recovered after acid hydrolysis with final values around 5 g_{xylose} /100 $g_{biomass}$. (Rojo et al., 2023) carried out acid and alkaline hydrolysis at 120 ºC to microalgal biomass grown on piggery wastewater obtaining also high monosaccharide recovery yield (60.7% of initial carbohydrates) by acid and high peptide recovery yield alkaline (81% of initial proteins) hydrolysis respectively. On the other hand, the LSD test confirmed significant differences ($\alpha < 0.05$) in the acid hydrolysis at 120 ºC for the recovery of peptides, increasing by 6% with doping biomass. For both chemical methods, peptide losses during the hydrolysis process were similar from NB and DB, and different peptide recoveries by acid hydrolysis are related to different protein solubilizations. The very low recovery of carbohydrates by alkaline hydrolysis, reduces the relevance of the significant ($\alpha < 0.05$) increase in glucose recovery up to 1.8 $g_{glucose}$ /100 $g_{biomass}$ with doping for this treatment. After the alkaline hydrolysis no xylose was recovered, but after acid hydrolysis the xylose losses were significantly higher from DB, so the presence of VA could promote xylose degradation.

Regarding physical and biological methods, the recoveries were lower than for chemical methods, obtaining the highest peptide recovery of 10.3 $g_{peptides}$ /100 $g_{biomass}$ with the UAEE-P from NB, followed by DB (7.8 $g_{peptides}$ /100 $g_{biomass}$), UAE from NB (7.1 $g_{peptides}$ /100 $g_{biomass}$) and from DB (6.5 $g_{peptides}$ /100 $g_{biomass}$). A certain effect of the presence of VA on peptide losses could be observed in the UAEE-P method ($\alpha < 0.05$) since peptide recovery decreased in the DB by 25%, while solubilization decreased by only 9%. The same increase on peptide losses occurred in the UAE and HE-P treatment but without significant differences in peptide recovery ($\alpha > 0.05$). On the other hand, glucose and xylose recoveries were reduced by doping with significant differences ($\alpha < 0.05$) in

some treatments, but the low amounts of recovered monosaccharides (< 2.7 g/100 $g_{biomass}$) make a detailed analysis of these differences uninteresting. It is worth mentioning only the increase on xylose losses by the presence of VA, resulting in lower xylose recovery from DB than from NB, contrary to the effect of doping on xylose solubilization.

3.7. Effect of TE on peptide and monosaccharide recovery

Fig. 3.B shows the recovery of peptides and the most abundant monosaccharides found in the algal biomass (glucose and xylose). As can be observed, again the chemical treatments at 120 ºC provided the highest recoveries and more specifically, the alkaline achieved a recovered amount of 27.3 $g_{peptides}$ /100 $g_{biomass}$ from NB, but with very low monosaccharides recoveries (< 2 g/100 $g_{biomass}$), while acid hydrolysis allowed for the recovery of high quantities of peptide (~26 $g_{peptides}$ /100 $g_{biomass}$ from both biomasses) and moderate recoveries of glucose (9.6 $g_{glucose}$ /100 $g_{biomass}$ from NB) and xylose (around 3.9 g_{xylose} /100 $g_{biomass}$). The LSD test ($\alpha < 0.05$) found significant effect of TE on peptide recovery after alkaline hydrolysis and on glucose recovery after acid hydrolysis. The decrease on peptide and glucose recoveries by TE doping, with values of 23.9 $g_{peptide}$ /100 $g_{biomass}$ by alkaline treatment and 5.8 $g_{glucose}$ /100 $g_{biomass}$ by acid treatment was related to differences on solubilization. No effect of TE on peptide and glucose losses was detected for chemical hydrolysis experiments.

Regarding physical and biological treatments, the notable influence that TE had on compounds recoveries can be observed in Fig. 4. B. The peptide recovery decreased in presence of these pollutants with significant differences ($\alpha < 0.05$), from 9.2 to 5 $g_{peptides}$ /100 $g_{biomass}$ with UAE, from 11.4 to 3.8 $g_{peptides}$ /100 $g_{biomass}$ with UAEE and from 6.6 to 1.1 $g_{peptides}$ /100 $g_{biomass}$ with HE-P. No effect of TE on peptide losses was found, being these differences again related to the effect of TE on solubilization, attributed to a possible enzyme inhibition (Finnegan and Chen, 2012; Maret, 2013). Finally, the recoveries of both monosaccharides were very low in all these experiments, being lower than 1 $g_{monosaccharide}$ /100 $g_{biomass}$ or even null from the DB after physical and biological treatments with significant differences due to TE doping ($\alpha < 0.05$) in the recoveries of glucose and xylose. The glucose and xylose losses in UAE, UAEE-P and HE-P treatments were higher from DB than from NB, and together with the lower solubilization, resulted in a remarkable decrease in the amount of recovered monosaccharides due to the presence of TE which could degrade them.

3.8. Effect of VA and TE on peptide and monosaccharide recovery

The recoveries of peptides, glucose, and xylose of assay 3 are illustrated in Fig. 4.C. The best results were achieved again by the chemical treatment with acid at 120 ºC, with significant differences between NB and DB ($\alpha < 0.05$) for peptides and glucose. The presence of these contaminants increased the peptide recovery from 14.9 $g_{peptides}$ /100 $g_{biomass}$ (NB) to 19.2 $g_{peptides}$ /100 $g_{biomass}$ (DB) and decreased the glucose recovery from 20.5 $g_{glucose}$ /100 $g_{biomass}$ (NB) to 11.9 $g_{glucose}$ /100 $g_{biomass}$ (DB). These variations are similar to those found in the solubilization values (increase of ~30% for proteins and reduction of ~43% for glucose), indicating that there was no effect of the contaminants on peptides and glucose losses in the acid hydrolysates. In the case of alkaline hydrolysis at 120 ºC, a significant effect ($\alpha < 0.05$) of VA and TE on peptide recovery was observed, increasing from 14.3 $g_{peptides}$ /100 $g_{biomass}$ (NB) to 19.5 $g_{peptides}$ /100 $g_{biomass}$ (DB). The difference between NB and DB peptide recovery in alkaline hydrolysis would be again due to the differences in solubilization which had similar percentage increases.

Regarding physical and biological treatments, the presence of VA and TE had a significant influence on the recoveries of peptides, glucose, and xylose ($\alpha < 0.05$) except for peptides in the UAE and HE-P. Peptide recovery after UAEE-P achieved 7.9 $g_{peptides}$ /100 $g_{biomass}$ from NB, and 4.7 $g_{peptides}$ /100 $g_{biomass}$ from DB, decreasing by a higher percentage than the solubilization values shown above (8%). The presence of VA and TE could promote the degradation of solubilized proteins, decreasing the efficiency of the UAEE-P treatment. On the other hand, the presence of VA and TE also decreased the monosaccharide recoveries achieving very low recoveries (0.24 $g_{monosaccharide}$ /100 $g_{biomass}$), highlighting the almost null values of xylose recovery in the three treatments. The presence of contaminants reduced monosaccharide recovery yields to a greater extent than solubilization yields. So, the combination of VA and TE favored the degradation of the released monosaccharides in UAE, UAEE-P and HE-P hydrolysates.

4. Conclusions

The presence of veterinary antibiotics and Cu, Zn and As increased the protein and decreased the carbohydrate contents of microalgal biomass grown on piggery wastewater. Biomass concentration in the photobioreactor also diminished due to stress by both pollutants. Protein solubilization and recovery increased in presence of VA and/or TE after chemical treatments but decreased after ultrasound and enzymatic treatments. Glucose solubilization was greatly reduced by VA and TE for all the studied treatments. The effect of contaminants on xylose solubilization was similar than on proteins, but they increased degradation reducing its recovery.

CRediT authorship contribution statement

Elena M Rojo: Writing – original draft, Formal analysis, Data curation. **Angel Alejandro Filipigh:** Methodology, Investigation, Formal analysis, Data curation. **Maria Hurtado:** Methodology, Investigation, Data curation. **Francisco Gabriel Acien:** Resources, Funding acquisition, Conceptualization. **Martina Ciardi:** Investigation, Data curation. **Silvia Bolado:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2024.103632.

References

Aggarwal, A., Sharma, I., Tripathi, B., Munjal, A., Baunthiyal, M., Sharma, V., 2011. Metal toxicity and photosynthesis. Photosynth.: Overv. Recent Prog. Future Perspect. 16, 229–236.

Amaro, H.M., Salgado, E.M., Nunes, O.C., Pires, J.C.M., Esteves, A.F., 2023. Microalgae systems - environmental agents for wastewater treatment and further potential biomass valorisation. J. Environ. Manag. 337, 117678 https://doi.org/10.1016/J.JENVMAN.2023.117678.

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative stress and antioxidant defense. World Allergy Organ. J. 5, 9–19. https://doi.org/10.1097/WOX.0B013E3182439613.

Chen, S., Wang, L., Feng, W., Yuan, M., Li, J., Xu, H., Zheng, X., Zhang, W., 2020. Sulfonamides-induced oxidative stress in freshwater microalga Chlorella vulgaris: evaluation of growth, photosynthesis, antioxidants, ultrastructure, and nucleic acids, 10 Sci. Rep. 2020 10 (1), 1–11. https://doi.org/10.1038/s41598-020-65219-2.

Ciardi, M., Gómez-Serrano, C., Lafarga, T., Acién, G., Llamas, B., Bolado, S., Fernández-Sevilla, J.M., 2022. Reduction in water consumption during the production of microalgae using diluted pig slurry in thin-layer cascade photobioreactors. J. Appl. Phycol. 34, 2905–2916. https://doi.org/10.1007/S10811-022-02826-8.

Collao, J., García-Encina, P.A., Blanco, S., Bolado-Rodríguez, S., Fernandez-Gonzalez, N., 2022. Current concentrations of Zn, Cu, and As in piggery wastewater compromise nutrient removals in microalgae–bacteria photobioreactors due to altered microbial communities. Biol. (Basel) 11. https://doi.org/10.3390/biology11081176.

Conde-Cid, M., Núñez-Delgado, A., Fernández-Sanjurjo, M.J., Álvarez-Rodríguez, E., Fernández-Calviño, D., Arias-Estévez, M., 2020. Tetracycline and sulfonamide antibiotics in soils: presence, fate and environmental risks, 1479 8, 1479 Processes 2020 Vol. 8. https://doi.org/10.3390/PR8111479.

Danouche, M., El Ghatchouli, N., Arroussi, H., 2022. Overview of the management of heavy metals toxicity by microalgae. J. Appl. Phycol. 2021 34 (1 34), 475–488. https://doi.org/10.1007/S10811-021-02668-W.

Finnegan, P.M., Chen, W., 2012. Arsenic toxicity: the effects on plant metabolism. JUN, 182 Front Physiol. 3. https://doi.org/10.3389/FPHYS.2012.00182.

Gomaa, M., Zien-Elabdeen, A., Hifney, A.F., Adam, M.S., 2021. Phycotoxicity of antibiotics and non-steroidal anti-inflammatory drugs to green algae Chlorella sp. and Desmodesmus spinosus: assessment of combined toxicity by Box–Behnken experimental design. Environ. Technol. Innov. 23, 101586 https://doi.org/10.1016/J.ETI.2021.101586.

Lee, J., Choi, O.K., Oh, D., Lee, K., Park, Y., Kim, D., 2020. Stimulation of lipid extraction efficiency from sewage sludge for biodiesel production through hydrothermal pretreatment, 13, 6392 Energies 2020 Vol. 13, 6392. https://doi.org/10.3390/EN13236392.

Leong, Y.K., Chang, J.S., 2020. Bioremediation of heavy metals using microalgae: recent advances and mechanisms. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2020.122886.

Li, X., Yang, C., Zeng, G., Wu, S., Lin, Y., Zhou, Q., Lou, W., Du, C., Nie, L., Zhong, Y., 2020. Nutrient removal from swine wastewater with growing microalgae at various zinc concentrations. Algal Res 46, 101804. https://doi.org/10.1016/J.ALGAL.2020.101804.

López-Pacheco, I.Y., Silva-Núñez, A., García-Perez, J.S., Carrillo-Nieves, D., Salinas-Salazar, C., Castillo-Zacarías, C., Afewerki, S., Barceló, D., Iqbal, H.N.M., Parra-Saldívar, R., 2021. Phyco-remediation of swine wastewater as a sustainable model based on circular economy. J. Environ. Manag. 278, 111534 https://doi.org/10.1016/J.JENVMAN.2020.111534.

López-Serna, R., García, D., Bolado, S., Jiménez, J.J., Lai, F.Y., Golovko, O., Gago-Ferrero, P., Ahrens, L., Wiberg, K., Muñoz, R., 2019. Photobioreactors based on microalgae-bacteria and purple phototrophic bacteria consortia: a promising technology to reduce the load of veterinary drugs from piggery wastewater. Sci. Total Environ. 692, 259–266. https://doi.org/10.1016/J.SCITOTENV.2019.07.126.

Manikandan, A., Suresh Babu, P., Shyamalagowri, S., Kamaraj, M., Muthukumaran, P., Aravind, J., 2022. Emerging role of microalgae in heavy metal bioremediation. J. Basic Microbiol 62, 330–347. https://doi.org/10.1002/JOBM.202100363.

Maret, W., 2013. Inhibitory zinc sites in enzymes. BioMetals 26, 197–204. https://doi.org/10.1007/S10534-013-9613-7.

Martin Juárez, J., Martínez-Páramo, S., Maté-González, M., García Encina, P.A., Muñoz Torre, R., Bolado Rodríguez, S., 2021. Evaluation of pretreatments for solubilisation of components and recovery of fermentable monosaccharides from microalgae biomass grown in piggery wastewater. Chemosphere 268, 129330. https://doi.org/10.1016/j.chemosphere.2020.129330.

Martín-Juárez, J., Vega-Alegre, M., Riol-Pastor, E., Muñoz-Torre, R., Bolado-Rodríguez, S., 2019. Optimisation of the production of fermentable monosaccharides from algal biomass grown in photobioreactors treating wastewater. Bioresour. Technol. 281, 239–249. https://doi.org/10.1016/j.biortech.2019.02.082.

Michelon, W., Matthiensen, A., Viancelli, A., Fongaro, G., Gressler, V., Soares, H.M., 2022. Removal of veterinary antibiotics in swine wastewater using microalgae-based process. Environ. Res 207, 112192. https://doi.org/10.1016/j.envres.2021.112192.

Naveed, S., Li, C., Lu, X., Chen, S., Yin, B., Zhang, C., Ge, Y., 2019. Microalgal extracellular polymeric substances and their interactions with metal(loid)s: a review. Crit. Rev. Environ. Sci. Technol. 49, 1769–1802. https://doi.org/10.1080/10643389.2019.1583052.

Rempel, A., Gutkoski, J.P., Nazari, M.T., Biolchi, G.N., Biduski, B., Treichel, H., Colla, L.M., 2022. Microalgae growth with a high concentration of emerging pollutants and phytotoxicity evaluation of cultivation wastewater. J. Water Process Eng. 46, 102616 https://doi.org/10.1016/J.JWPE.2022.102616.

Rempel, A., Nadal Biolchi, G., Farezin Antunes, A.C., Gutkoski, J.P., Treichel, H., Colla, L.M., 2021. Cultivation of microalgae in media added of emergent pollutants and effect on growth, chemical composition, and use of biomass to enzymatic hydrolysis. Bioenergy Res 14, 265–277. https://doi.org/10.1007/S12155-020-10177-W.

Rojo, E.M., Filipigh, A., Moldes, D., Vega, M., Bolado, S., 2022. Potential of microalgae for protein production. Algal Biorefineries Circ. Bioecon. 91–132. https://doi.org/10.1201/9781003195405-4.

Rojo, E.M., Filipigh, A.A., Bolado, S., 2023. Assisted-enzymatic hydrolysis vs chemical hydrolysis for fractional valorization of microalgae biomass. Process Saf. Environ. Prot. https://doi.org/10.1016/j.psep.2023.03.067.

Rojo, E.M., Molinos-Senante, M., Filipigh, A.A., Lafarga, T., Fernández, F.G.A., Bolado, S., 2023. Agricultural products from algal biomass grown in piggery wastewater: a techno-economic analysis. Sci. Total Environ. 887, 164159 https://doi.org/10.1016/J.SCITOTENV.2023.164159.

Rojo, E.M., Piedra, I., González, A.M., Vega, M., Bolado, S., 2021. Effect of process parameters on the valorization of components from microalgal and microalgal-bacteria biomass by enzymatic hydrolysis. Bioresour. Technol., 125256 https://doi.org/10.1016/j.biortech.2021.125256.

Roy, J., 2011. The stability of medicines. Introd. Pharm. Sci. 153–181. https://doi.org/10.1533/9781908818041.153.

Salama, E.S., Roh, H.S., Dev, S., Khan, M.A., Abou-Shanab, R.A.I., Chang, S.W., Jeon, B.H., 2019. Algae as a green technology for heavy metals removal from various wastewater. World J. Microbiol Biotechnol. 35, 1–19. https://doi.org/10.1007/S11274-019-2648-3.

Sánchez-zurano, A., Rodríguez-miranda, E., Guzmán, J.L., Acién-fernández, F.G., Fernández-sevilla, J.M., Grima, E.M., 2021. ABACO: a new model of microalgae-bacteria consortia for biological treatment of wastewaters, 11, 998 Appl. Sci. 2021 Vol. 11, 998. https://doi.org/10.3390/APP11030998.

Singh, D.V., Bhat, R.A., Upadhyay, A.K., Singh, R., Singh, D.P., 2021. Microalgae in aquatic environs: a sustainable approach for remediation of heavy metals and emerging contaminants. Environ. Technol. Innov. 21, 101340 https://doi.org/10.1016/J.ETI.2020.101340.

Smith, D.R., Maroney, M.J., Nordberg, M., Tyson, J.F., 2022. General chemistry of metals, sampling, analytical methods, and speciation. Handb. Toxicol. Met.: Vol. I: Gen. Consid. 15–54. https://doi.org/10.1016/B978-0-12-823292-7.00001-2.

Tejirian, A., Xu, F., 2010. Inhibition of cellulase-catalyzed lignocellulosic hydrolysis by iron and oxidative metal ions and complexes. Appl. Environ. Microbiol 76, 7673–7682. https://doi.org/10.1128/AEM.01376-10.

Upadhyay, A.K., Singh, R., Singh, D.V., Singh, L., Singh, D.P., 2021. Microalgal consortia technology: a novel and sustainable approach of resource reutilization, waste management and lipid production. Environ. Technol. Innov. 23, 101600 https://doi.org/10.1016/J.ETI.2021.101600.

Urrutia, C., Yañez-Mansilla, E., Jeison, D., 2019. Bioremoval of heavy metals from metal mine tailings water using microalgae biomass. Algal Res 43, 101659. https://doi.org/10.1016/J.ALGAL.2019.101659.

Van Epps, A., Blaney, L., 2016. Antibiotic residues in animal waste: occurrence and degradation in conventional agricultural waste management practices. Curr. Pollut. Rep. 2016 2 (3 2), 135–155. https://doi.org/10.1007/S40726-016-0037-1.

Wang, Y., Li, J., Lei, Y., Li, X., Nagarajan, D., Lee, D.J., Chang, J.S., 2022. Bioremediation of sulfonamides by a microalgae-bacteria consortium – analysis of pollutants removal efficiency, cellular composition, and bacterial community. Bioresour. Technol. 351, 126964 https://doi.org/10.1016/J.BIORTECH.2022.126964.

Wang, Y., Sutton, N.B., Zheng, Y., Dong, H., Rijnaarts, H.H.M., 2023. Seasonal variation in antibiotic resistance genes and bacterial phenotypes in swine wastewater during three-chamber anaerobic pond treatment. Environ. Res 216, 114495. https://doi.org/10.1016/J.ENVRES.2022.114495.

Zambrano, J., García-Encina, P.A., Hernández, F., Botero-Coy, A.M., Jiménez, J.J., Irusta-Mata, R., 2023. Kinetics of the removal mechanisms of veterinary antibiotics in synthetic wastewater using microalgae–bacteria consortia. Environ. Technol. Innov. 29, 103031 https://doi.org/10.1016/J.ETI.2023.103031.

Zambrano, J., García-Encina, P.A., Hernández, F., Botero-Coy, A.M., Jiménez, J.J., Irusta-Mata, R., 2021. Removal of a mixture of veterinary medicinal products by adsorption onto a Scenedesmus almeriensis microalgae-bacteria consortium. J. Water Process Eng. 43, 102226 https://doi.org/10.1016/J.JWPE.2021.102226.

Zheng, S., Zhang, G., Wang, H.J., Long, Z., Wei, T., Li, Q., 2021. Progress in ultrasound-assisted extraction of the value-added products from microorganisms. World J. Microbiol Biotechnol. 37, 1–14. https://doi.org/10.1007/S11274-021-03037-Y.

Zhou, T., Wang, J., Zheng, H., Wu, X., Wang, Y., Liu, M., Xiang, S., Cao, L., Ruan, R., Liu, Y., 2018. Characterization of additional zinc ions on the growth, biochemical composition and photosynthetic performance from Spirulina platensis. Bioresour. Technol. 269, 285–291. https://doi.org/10.1016/J.BIORTECH.2018.08.131.