Ecotoxicological response of *Spirulina platensis* to coexisted copper and zinc in anaerobic digestion effluent

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Abstract

Copper ion (Cu^{2+}) and zinc ion (Zn^{2+}) are widely co-existent in anaerobic digestion effluent as typical contaminants. This work aims to explore how Cu^{2+}-Zn^{2+} association affects physiological properties of *S. platensis* using Schlösser medium (SM) and sterilized anaerobic digestion effluent (SADE). Microalgae cells viability, biochemical properties, uptake of Cu^{2+} and Zn^{2+}, and risk assessment associated with the biomass reuse as additives to pigs were comprehensively assessed. Biomass production ranged from 0.03 to 0.28 g/L in SM and 0.63 to 0.79 g/L in SADE due to the presence of Cu^{2+} and Zn^{2+}. Peak value of chlorophyll-a and carotenoid content during the experiment decreased by 70-100% and 40-100% in SM, and by 70-77% and 30-55% in SADE. Crude protein level reduced by 4-41% in SM and by 65-75% in SADE. The reduction ratio of these compounds was positively related to the Cu^{2+} and Zn^{2+} concentrations. Maximum value of saturated and unsaturated fatty acids was both obtained at 0.3 Cu+2.0 Zn (50.8% and 22.8%, respectively) and 25% SADE reactors (33.8% and 27.7%, respectively). Uptake of Cu in biomass was facilitated by Zn^{2+} concentration (> 4.0 mg/L). Risk of *S. platensis* biomass associated with Cu^{2+} was higher than Zn^{2+}. *S. platensis* from SM ($Cu^{2+} \leq 0.3$ mg/L and $Zn^{2+} \leq 4.0$ mg/L) and diluted SADE (25% and 50% SADE) reactors could be used as feed additives without any risk (hazard index < 1), which provides sufficient protein and fatty acids for pig consumption. These results revealed the promising application of using *S. platensis* for bioremediation of Cu^{2+} and Zn^{2+} in anaerobic digestion effluent and harvesting biomass for animal feed additives.

Keywords: *S. platensis*; Heavy metal; Sterilized anaerobic digestion effluent; Ecotoxicological response; Risk assessment

1. Introduction

Anaerobic digestion effluent (ADE) from intensive livestock industry contains high concentrations of nitrogen (N) and phosphates (P) along with heavy metals such as copper ion (Cu^{2+}) and zinc ion (Zn^{2+}), and the concentrations of these pollutants sometimes even exceed the environmental regulations (Baker et al., 2021; Cao et al., 2018). The inappropriate disposal of the ADE can cause serious consequences, such as eutrophication, pathogen contamination, ecosystem deterioration and so on (Praveen et al., 2018). The heavy metals, such as Cu^{2+} and Zn^{2+}, released into environment can migrate to groundwater and surface waters, and even accumulated in soils and crops, exerting a profound and negative effect on environmental and human health (Feng et al., 2018). Therefore, it is necessary to remove those nutrients and heavy metals from ADE before they are released into the environment (Cai et al., 2013; Gupta et al., 2022). Several techniques, i.e., membrane bioreactor, chemical precipitation, and constructed wetlands, have been developed for ADE treatment but all facing eco-friendly and sustainable problems or difficulties (such as high cost, energy consumption, low efficiency and so on) (Monfet et al., 2017).

Microalgae-based technology shows a promising remedy of ADE in nutrient recovery, which converts N and P in ADE into biomass for future utilization (Hasan et al., 2021; Xie et al., 2018; Wang et al., 2019a,b). Ammonia nitrogen (NH_4^+-N) in the ADE can act as a major nitrogen source for the synthesis of protein, enzymes and energy transfer, and P can be assimilated for the production of phospholipids, nucleic acid, and ATP during microalgae growth (Cheng et al., 2020). A variety of microalgae has been applied for ADE treatment including *Desmodesmus* sp., *Chlorella* sp. and *Scenedesmus* sp.. Previous reports documented that above 90% NH_4^+-N and 84% total nitrogen from

ADE were eliminated by *Desmodesmus* sp. after 14 days of cultivation (Li et al., 2021); while 93% NH_4^+-N and 100% phosphate (PO_4^{3-}) from ADE were removed using a microalgae co-culture (*Chlorella* sp. and *Scenedesmus* sp.) (Hasan et al., 2021). Among multiple microalgae used for ADE treatment, *S. platensis* attracted the attention of researchers due to its fast growth rate, strong adaptability, high-valuable metabolites accumulation (Zhou et al., 2021; Zhou et al., 2018). With *S. platensis*, up to 99% NH_4^+-N and 45% PO_4^{3-} were assimilated to further convert into biomass with the supplementation of HCO_3^- in ADE, leading to a biomass concentration of 1.50 g/L after 12 days (Matos et al., 2021). Compared to other microalgae, *S. platensis* also has the ability to auto-flocculate and float on the water surface owing to the filamentous structure, which facilitates the biomass harvest for further utilization (Razak and Sharip, 2020). It is worth noting that *S. platensis* is the most popular food supplement and animal feeds around the world with the richest source of plant proteins (60 - 70% of its weight) among microalgae biomass (Usharani, 2012). As stated above, the merits of nutrient recovery, biomass productivity and value-added metabolites potential make microalgae-based technology a more appealing technique for ADE purification.

Aside from nutrients, ADE also possess high concentrations of Cu^{2+} (< 5 mg/L) and Zn^{2+} (< 10 mg/L) (Jin and Chang, 2011). Although Cu^{2+} and Zn^{2+} are essential microelements for microalgal growth in pure cultivation, the excess of these heavy metals shows great inhibition for microalgae growth and metabolism, which greatly affect biomass production and nutrient conversion (El.Din, 2017; Zhou et al., 2018). Our previous study observed that the increase of Zn^{2+} concentration from 0.0 to 8.0 mg/L in culture medium reduced the biomass production of *S. platensis* by 70.3% (Zhou et al., 2018). Increase of Cu^{2+} concentration from 0.5 to 3 mg/L also decreased biomass yield of *S. platensis* (El.Din, 2017). In ADE with *Coelastrella* sp., increase of Cu^{2+}

concentration from 0.0 to 3.0 mg/L decreased the removal efficiency of NH_4^+-N from 80% to 39% (Li et al., 2018), but increased Zn^{2+} from 0.0 to 2.0 mg/L reduced total phosphate (TP) removal efficiency by 7.7% (Li et al., 2020). More importantly, Cu^{2+} and Zn^{2+} are usually co-existed in ADE. de Souca Oliveira et al. (2021) investigated the simultaneous interferences of Cu^{2+} and Zn^{2+} in high-rate algal ponds (HRAP) and found lowest biomass yield of *Chlorella* sp. was observed in HRAP with 0.5 mg/L Cu^{2+} and 5.0 mg/L Zn^{2+}. To date, the impact of both Cu^{2+} and Zn^{2+} in pure culture or ADE on *S. platensis* growth remains unclear. Furthermore, Cu^{2+} and Zn^{2+} exposure also alter the biochemical properties of microalgae biomass. The inhibition of total chlorophyll and protein content in *S. platensis* biomass caused by 3 mg/L Cu^{2+} were 65.9% and 63.0%, respectively (El.Din, 2017). The increase of Zn^{2+} from 2.0 to 8.0 mg/L inhibited the biosynthesis of photosynthetic pigments (phycocyanin, chlorophyll-a and carotenoid) but improved the amount of polyunsaturated fatty acids (PUFA) (C18:2 and C18:3) in *S. platensis* biomass (Zhou et al., 2018). Rocha et al. (2021) reported that the amounts of total lipids and fatty acids in the biomass of *Selenastrum gracile* was enhanced linearly by with the concentration of Cu^{2+} from 0.7×10^{-2} to 9.6×10^{-2} μM in culture medium, whereas the amount of saturated fatty acids declined. During the cultivation of *Coelastrella* sp. in the swine wastewater, high Zn^{2+} concentration (> 1.0 mg/L) decreased chlorophyll-a content but increased protein level against Zn^{2+} toxicity (Li et al., 2020), whereas both chlorophyll-a and protein content decreased at 0.1 - 3 mg/L Cu^{2+} (Li et al., 2018). Indeed, the growth and response of microalgae varies between microalgae species exposed to Cu^{2+}- Zn^{2+} association. *S. platensis* is considered as a good source of nutrient in animal diet due to the abundant proteins, fatty acids and carotenoid (Zhou et al., 2018). The alteration in the content of those nutrients in biomass may limit the application as feed additives. Up to now, the change of biochemical

properties of *S. platensis* biomass due to the presence of Cu^{2+} and Zn^{2+} in ADE has not been well investigated. In addition, *S. platensis* has been demonstrated to absorb Cu^{2+} and Zn^{2+} from aquatic environments (Chan et al., 2013). The uptake capacity ranged from 0.6 to 78.0 mg/g dry weight of Cu^{2+} (Nalimova et al., 2005; Vannela and Verma, 2006) and 0.1 to 38.7 mg/g dry weight of Zn^{2+} (Pane et al., 2008; Vannela and Verma, 2006). However, the uptake capacity of Cu^{2+} or Zn^{2+} may be impacted by the presence of other metals. Franklin et al. (2002) reported that Cu^{2+} hindered the uptake of Zn^{2+} by *Chlorella* sp., while Zn^{2+} had no obvious impact on the uptake of Cu^{2+}. Those metals accumulated in metal-rich biomass can enter the food chain and result in serious health issues in animals (Nagarajan et al., 2021). The uptake capacity of *S. platensis* to Cu^{2+} and Zn^{2+} in Cu^{2+}-Zn^{2+} association system, and whether heavy metals-enriched biomass can be reused as supplemented animal feed additives are still not investigated.

Although several heavy metals (i.e., Cu^{2+}, Zn^{2+}, Cr^{6+}, Cd^{2+}, and Pb^{2+}) are found in swine manure, reaching different concentrations in the ADE; concentrations of Cu^{2+} and Zn^{2+} are found 10-100 times higher than other metals due to their widespread occurrence in feed additives and incomplete metabolism in pigs (do Amaral et al., 2014; Feng et al., 2018; Zhang et al., 2016). Therefore, Cu^{2+} and Zn^{2+} were chosen as the main representatives of heavy metals in ADE in this study to evaluate their impacts on *S. platensis*. This study aims to evaluate the impacts of Cu^{2+} and Zn^{2+} association on the biomass production and reuse from ADE using *S. platensis*. Microalgae cells viability, biochemical properties of biomass (i.e., photosynthetic pigments, protein, and fatty acids), accumulation of Cu^{2+} and Zn^{2+} in biomass and risk assessment associated with the biomass reuse as an additive to pigs were comprehensively assessed under laboratory tests with eight concentration scenarios. The results improved the understanding of the impact of Cu^{2+} and Zn^{2+} association on the development of

microalgae and environment and provided guidance for the treatment of ADE using *S. platensis*.

2. Materials and methods

2.1 Microalgae strain

Spirulina platensis (FACHB: GY-D18), was procured from the Institute of Hydrobiology Chinese Academy of Science, PR China. The microalgal strain was maintained axenically in 3 L Erlenmeyer flasks containing 1.5 L of Schlösser medium (SM) (Schlösser, 1982) with the composition provided in Table S1 at 25±1 °C with a continuous light intensity of about 1200 Lux. The flasks were manually shaken three times daily. After 7 days of cultivation, *S. platensis* suspension was centrifuged at 4000 rpm for 10 min at ambient temperature and washed three times using deionized water. Afterwards, microalgae cells were re-suspended in sterile SM for inoculation.

2.2 Growth of S. platensis in SM with Cu^{2+}-Zn^{2+} binary metal treatment

The effects of Cu^{2+}-Zn^{2+} association concentrations in the SM on *S. platensis* development were evaluated using a series of tests. 50 mL of *S. platensis* was cultured in 500 mL Erlenmeyer flasks containing 300 mL SM, which were used as reactors for further Cu^{2+}-Zn^{2+} binary metal treatment. In most ADE, the concentrations of Cu^{2+} was < 5 mg/L and Zn^{2+} was < 10.0 mg/L (Jin and Chang, 2011). To mimic the Cu^{2+}-Zn^{2+} association concentrations in ADE, copper sulfate ($CuSO_4 \cdot 5H_2O$) (98% purity, Aladdin Biochemical Technology Co., Ltd, China) and zinc sulfate ($ZnSO_4 \cdot 7H_2O$) (99% purity, Aladdin Biochemical Technology Co., Ltd, China) were added to the reactors to achieve eight different concentration scenarios (Table 1). The reactors with SM only (without the addition of Cu^{2+} and Zn^{2+}) were used as control ($Control_{SM}$). Triplicate reactors

under each concentration scenario were incubated for 11 days under 25±1 °C and light intensity of about 1200 Lux. The reactors were shaken manually three times a day.

The growth performance of *S. platensis* was calculated using the biomass concentrations. Chlorophyll fluorescence activity was measured every two days to reflect the photosynthetic activity of *S. platensis* cells under different concentration scenarios. Photosynthesis pigments of *S. platensis* were reflected by chlorophyll-a and carotenoid concentrations, which were measured every two days during the cultivation. Biochemical components (crude protein and fatty acids) and uptake of Cu^{2+} and Zn^{2+} were also measured after the harvest of *S. platensis* biomass on the 11th day. The details of the analytical process were described in section 2.4.

2.3 Growth of S. platensis in presence of sterilized anaerobic digestion effluent with Cu^{2+}-Zn^{2+}

ADE was collected from a pig farm located in Fengcheng City, Jiangxi Province, China. The ADE was centrifuged at 4 ºC and 10,000 rpm for 15 min to collect the supernatant. To minimize the contribution of other microorganisms in the ADE, the supernatant was sterilized at 121 ºC for 20 min. The main characteristics of sterilized anaerobic digestion effluent (SADE) were determined and shown in Table 2. SADE was further diluted in two different concentrations (25% and 50%) using deionized water, which were labelled as 25% SADE and 50% SADE, respectively. Dilution ratios of ADE were ascertained as our previous pre-experiment studies due to the obvious difference in growth and chlorophyll fluorescence activity (data not shown). 250 mL of 25%, 50% and 100% (no dilution) SADE was transferred in sterilized 500 mL Erlenmeyer flasks for *S. platensis* cultivation and each SADE concentration was prepared in triplicates.

The responding mechanisms of *S. platensis* in SADE containing Cu^{2+} and Zn^{2+} were assessed. 50 mL of *S. platensis* was incubated in 500 mL Erlenmeyer flasks containing different ratios of SADE as described above for 10 days and 250 mL of SM was served as a control without the addition of Cu^{2+} and Zn^{2+} ($Control_{SADE}$). The concentration of magnesium ion (Mg^{2+}) in 100% SADE was comparable with that of SM reactors, which prevented the effect caused by Mg^{2+}. Light and temperature conditions were maintained at about 1200 Lux and 25±1 °C, respectively. All the tests were shaken manually three times daily. During the cultivation period, various biological activity parameters, including chlorophyll fluorescence activity on the 2, 4, 6, 8, and 10 day, chlorophyll-a and carotenoid concentrations on the 2, 5, 8, and 10 day. Crude protein, fatty acids, and uptake of Cu^{2+} and Zn^{2+} in harvested *S. platensis* biomass were also measured with the analytical details provided in section 2.4.

2.4 Analytical methods and statistical analysis

Biomass concentrations of *S. platensis* during the cultivation period were assessed by measuring the dry cell weight (DCW) of microalgae biomass at 105 °C for 24 h according to the method described in Zhou et al. (2021). The growth inhibition rate (GIR) of *S. platensis* was calculated as follows:

$$GIR\ (\%) = [(DCW_{control} - DCW_{sample}) / DCW_{control}] \times 100\% \quad (1)$$

where DCW_{sample} represents the dry cell weight (g/L) of biomass in reactors with SM or SADE with the presence of Cu^{2+} and Zn^{2+}, and $DCW_{control}$ means the dry cell weight (g/L) of biomass in corresponding control, such as $Control_{SM}$ and $Control_{SADE}$.

The maximum photochemical quantum yield of Photosystem Ⅱ (PS Ⅱ) (F_v/F_m) was used to represent chlorophyll fluorescence activity. F_v/F_m was determined in dark using Water-PAM (WALZ, Germany) and obtained from the measured chlorophyll

fluorescence induction curves developed by WinControl software v.3.2. F_v/F_m is usually calculated as follows (Ramanna et al., 2014):

$$F_v = F_m - F_0 \quad (2)$$

$$F_v / F_m = (F_m - F_0) / F_m \quad (3)$$

where F_v is the variable fluorescence. F_m and F_0 are the maximum fluorescence and initial chlorophyll fluorescence measured of *S. platensis* in dark, respectively.

An ultraviolet spectrophotometer was employed to determine the chlorophyll-a and carotenoid concentration in *S. platensis* biomass through spectrophotometry in the process of cultivation as described previously (Zhou et al., 2018). Protein content of *S. platensis* was presented as crude protein (TN×6.25), which was estimated from total nitrogen (TN) using a conversion factor (6.25) (Zhou et al., 2021). Fatty acids profile was analyzed through GC-MS (Agilent 7890B-7000D, USA) as discussed in the previous study (Zhou et al., 2018).

Cu and Zn contents in the freeze-dried *S. platensis* biomass were determined by ICP-OES (Optima 8000, United States), respectively. Samples were digested with HNO_3 (70% purity, Aladdin Biochemical Technology Co., Ltd, China) and HCl (37% purity, Aladdin Biochemical Technology Co., Ltd, China) mixture (HNO_3:HCl = 1:3, v/v) in a microwave oven and the procedure was as follows: (1) at 120 °C for 2 min, (2) at 160 °C for 3 min, (3) at 180 °C for 20 min. Afterwards, samples were diluted to 1:50 with ultra-pure water before measurement. Then the diluted sample was analyzed by ICP-OES (Zhou et al., 2018).

2.5 Risk assessment

The risk of pig exposure to Cu and Zn after ingesting these microalgae biomass was

assessed by the average daily intake (ADI) (mg/(kg·day)) and the hazard quotient (HQ) as equations below Eq (4) and (5) (Shamsollahi et al., 2019):

$$ADI = (C \times I \times EF \times ED) / (BW \times AT) \quad (4)$$

where C means the Cu or Zn concentrations in microalgae (mg/kg); I is the ingestion rate of biomass (kg/day), 0.075 kg/day for one pig in this study (Hugh et al., 1985; Michalak et al., 2015); EF means the exposure frequency (days/years), in this study, 87 day/years (Michalak et al., 2015); ED means the exposure duration (year), 1 year for this study; BW means the body weight of the exposed individual (kg), 30 kg for pigs in this study (Šimkus et al., 2013); and AT means the exposure time period (day), ED×365 days (Zheng et al., 2010).

$$HQ = ADI / RfD \quad (5)$$

where RfD represents the reference dose for ingestion (mg/(kg·day)). Daily requirements of Cu and Zn are 6.01 mg Cu and 90.2 mg for one pig weighing 25 - 50kg, respectively (National Research Council, 2012). Thus, RfD was 0.12 mg/(kg·day) for Cu and 1.80 mg/(kg·day) for Zn, derived by dividing the daily requirements of heavy metals by body weight (50 kg).

For a mixture of pollutants, the hazard index (HI) is the summation of hazard quotients for all heavy metals in this study, and is calculated by Eq. (6):

$$HI = HQ_{Cu} + HQ_{Zn} \quad (6)$$

where HQ_{Cu} and HQ_{Zn} mean the HQ value of Cu and Zn, respectively.

The statistical analysis of all data and data plotting were performed with Microsoft Excel 2013 and Origin 2017. The comparison was performed by One-Way ANOVA using IBM SPSS Statistics 26. The $P < 0.05$ was considered as statistically significant.

3. Results

3.1 Biomass growth

3.1.1 Biomass growth in SM

Fig.1 shows the growth and chlorophyll fluorescence activity (F_v/F_m) of *S. platensis* cell at various treatments for 11 days of cultivation. The presence of Cu^{2+} and Zn^{2+} in SM greatly reduced the biomass development of *S. platensis* (Fig 1A). In control reactors, biomass concentration increased steadily to 0.62 g/L after 11 days of cultivation. In SM with the presence of Cu^{2+} or Zn^{2+}, the biomass was 0.03 - 0.28 g/L after 11 days. This biomass reduction is directly related to the increase of Cu^{2+} or Zn^{2+} concentration. In SM with the presence of 0.3 mg/L Cu^{2+}, the increase of Zn^{2+} from 0 to 8.0 mg/L reduced biomass concentrations from 0.28 g/L to 0.03 g/L. Increased Cu^{2+} concentrations from 0.3 to 0.6 mg/L at constant Zn^{2+} levels in SM also resulted in significant decrease of biomass yield from 0.22 - 0.28 g/L to 0 - 0.12 g/L ($P < 0.05$). In particular, the growth at 0.6 Cu+2.0 Zn was almost completely inhibited. Moreover, extended lag phases (from 0 day to 3 - 9 days) were observed in the reactors with increased Zn^{2+} level at 0.3 mg/L Cu^{2+} or increased Cu^{2+} level at constant Zn^{2+} concentration. These results indicate the growth of *S. platensis* was suppressed significantly ($P < 0.05$) in SM with the addition of Cu^{2+} and Zn^{2+}. This is also reflected by the growth inhibition rate (GIR) shown in Fig. 1C. When Zn^{2+} concentrations increased from 2.0 to 8.0 mg/L under 0.3 mg/L Cu^{2+}, GIR increased from 63.9% to 95.9% on the 11th day, which was much higher than the GIR at 0.3 Cu^{2+} (55.5%). Under 0.6 mg/L Cu^{2+}, the addition of Zn^{2+} also enhanced the GIR from 81.4% to 99.8%. Meanwhile, an increased GIR from 55.5% to 99.8% was also observed with increasing Cu^{2+} concentration (from 0.3 to 0.6 mg/L) under a fixed Zn^{2+} concentration (0.0 or 2.0 mg/L) on the 11th day.

F_v/F_m is widely used as an indicator of chlorophyll fluorescence activity to evaluate the toxicity of pollutants on microalgae, and the F_v/F_m value greater than 0.5 is generally associated with an acceptable physiological acclimation of algae to environmental conditions (Ramanna et al., 2014). F_v/F_m value differed when *S. platensis* suffered heavy metal stress (Fig. 1E). In control reactors, F_v/F_m value improved steadily and reached about 0.7 on the 11th day. In line with the trend of biomass concentrations, F_v/F_m value decreased with the increased Zn^{2+} levels from 0.0 to 8.0 mg/L at 0.3 mg/L Cu^{2+} concentration in SM, ranging from 0.5 to 0.7 at the end of the cultivation, indicating *S. platensis* was acclimatized in such conditions. An apparent negative correlation was also observed between Cu^{2+} level and F_v/F_m value at fixed Zn^{2+} levels in SM, indicating increase of Cu^{2+} levels at constant Zn^{2+} in SM reduced chlorophyll fluorescence activity of *S. platensis*.

3.1.2 Biomass growth in SADE

Biomass yield of *S. platensis* exhibited a continual upward trend for 10 days cultivation, obtaining 0.99 g/L in control reactors and 0.63 - 0.79 g/L in reactors with SADE (Fig. 1B). Biomass concentration in 100% SADE enhanced obviously to 0.45 g/L in the first day while the biomass yield in 25% and 50% SADE was significantly lower than 100% SADE (Fig. 1B). It is likely there were more abundant nutrients in 100% SADE reactors than diluted SADE, which could be consumed by *S. platensis* for biomass accumulation. It was found that higher SADE proportions in the reactors led to lower GIR in the first 3 days, corresponding to -304.9% to -106.2% in 100% SADE reactors, -107.1% to -15.8% in 50% SADE reactors and -42.2% to -15.8% in 25% SADE (Fig. 1D), implying promotion effect of SADE on *S. platensis* growth at the start of the experimentation. After 3 days, GIR increased slowly and then tended to be stable of 21.8% in 25% SADE,

20.3% in 50% SADE and 36.5% in 100% SADE. However, F_v/F_m value in 100% SADE reactors significantly reduced to 0.2 ($P < 0.05$) (Fig. 1F), suggesting the growth of *S. platensis* cells was inhibited. F_v/F_m value ranged from 0.5 to 0.6 in 25% SADE reactors but from 0.3 to 0.5 in 50% SADE reactors, implying *S. platensis* cells was not acclimatized in 50% SADE reactors.

3.2 Accumulation of intracellular biochemical components

3.2.1 Photosynthetic pigments

Concentrations of photosynthetic pigments in different reactors are displayed in Fig. 2. In $Control_{SM}$, the chlorophyll-a and carotenoid concentrations of *S. platensis* biomass gradually increased during the cultivation, reaching the maximum values at 3.49 mg/L and 1.51 mg/L on the 10^{th} day, respectively (Fig. 2A and 2B). The production of chlorophyll-a and carotenoid was completely inhibited at 0.6 Cu+2.0 Zn on the 10^{th} day. Increase of Cu^{2+} concentration from 0.3 to 0.6 mg/L at fixed Zn^{2+} reduced chlorophyll-a and carotenoid levels by 32.5 - 100% and 49.3 - 100% on the 10^{th} day, respectively. When Zn^{2+} concentration was elevated from 0.0 mg/L to 8.0 mg/L at 0.3 mg/L Cu^{2+}, maximum chlorophyll-a and carotenoid levels increased from 0.77 and 0.67 mg/L to 1.03 and 0.91 mg/L and then decreased to 0.13 and 0.16 mg/L, respectively. The peak values of chlorophyll-a and carotenoid were achieved at 0.3 Cu+4.0 Zn and 0.3 Cu+2.0 Zn, respectively, which were significantly lower than that in $Control_{SM}$ ($P < 0.05$). This implies the Cu^{2+} and Zn^{2+} in SM can alter the biosynthesis of chlorophyll-a and carotenoid in *S. platensis*. More importantly, the presence of Cu^{2+} and Zn^{2+} in SM accelerated the time required to reach the peak of chlorophyll-a and carotenoid levels. In $Control_{SM}$, the peak reached on the 10th day. Increase of Zn^{2+} from 0.0 to 8.0 mg/L at 0.3 mg/L Cu^{2+} and increase of Cu^{2+} from 0.3 to 0.6 mg/L at fixed Zn^{2+} concentrations

both reduced the time required for the peak of chlorophyll-a and carotenoid levels from 10 days to 4 days.

As depicted in Fig. 2C and 2D, chlorophyll-a and carotenoid concentrations in *S. platensis* cells accumulated with the cultivation time and reached a peak value of 10.17 mg/L and 2.87 mg/L on day 10 in $Control_{SADE}$, respectively. Similarly, for 50% SADE and 100% SADE, the production of chlorophyll-a and carotenoid increased slowly and reached a peak value on day 10. In comparison to $Control_{SADE}$, the peak values of chlorophyll-a and carotenoid decreased by 70.7 - 77.2% and 38.8 - 55.4% in 50% SADE and 100% SADE, respectively. In the reactors with 25% SADE, the maximum value of chlorophyll-a and carotenoid declined by 70.0% and 30.3% in comparison to $Control_{SADE}$, respectively. As stated above, the increase of SADE content clearly reduced the production of chlorophyll-a and carotenoid.

3.2.2 *Biochemical composition of microalgae biomass*

The biochemical compositions of *S. platensis* biomass can be reflected in crude protein contents and fatty acids profiles (Fig. 3). In SM, the presence of Cu^{2+} and Zn^{2+} significantly reduced the production of crude protein in *S. platensis* biomass by 5.0 - 41.1% ($P < 0.05$) (Fig. 3A) in comparison to $Control_{SM}$. Under the same Cu^{2+}, the increase of Zn^{2+} from 0.0 to 8.0 mg/L reduced the production of crude protein from 70.0% to 43.4%. Under the same Zn^{2+}, the increase of Cu^{2+} (0.3 - 0.6 mg/L) reduced the synthesis of crude protein from 70.0% to 46.9%. Similarly, in reactors with SADE, regardless of the dilution ratio of SADE, the crude protein production in *S. platensis* biomass decreased by 65.1 - 75.3% in comparison to $Control_{SADE}$ (Fig. 3C). A higher dilution ratio of SADE contributed to a slight increase of 36.1% - 41.4% in crude protein levels in comparison to 100% SADE.

Fig. 3B and 3D reveal the compositional distribution of fatty acids extracted from *S. platensis* biomass, where C16-C18 were the main components. The fatty acids profiles of *S. platensis* cells at 0.3 Cu+8.0 Zn and 0.6 Cu+2.0 Zn were not detected due to the extremely low biomass. In SM reactors, C16:0 was dominant and followed by C18:0, C18:2, C18:3, C18:1, and C16:1 (Fig. 3B). Unsaturated fatty acids (UFA) are composed of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Despite C16:0 is the predominant fatty acids in SM, most interest is focused on the polyunsaturated fatty acids (PUFA) with two or more double bonds (such as γ-linolenic acid) due to a wide range of application in food additives (Tonietto et al., 2014). The amount of SFA and UFA in $Control_{SM}$ were 41.5% and 19.0%, respectively, where the fraction of PUFA was about double as abundant as MUFA. The presence of Cu^{2+} and Zn^{2+} in SM exhibited negligible impact on MUFA amount, and altered the fraction of SFA, PUFA, and UFA. With the increase of Cu^{2+} concentration from 0.0 to 0.6 mg/L in the absent Zn^{2+} of SM, the proportion of SFA (mainly C16:0) and UFA (mainly C18:2 and C18:3) decreased by 14.0% and 29.3%, respectively. This implies that Cu^{2+} could decrease the saturation of C16 and C18, which was opposite to the conclusion of Li et al. (2018) that the proportion of C16:0 and C18:0 increased with the elevated Cu^{2+} concentration (1-3 mg/L) in anaerobically digested swine wastewater (ADSW). With the increase of Zn^{2+} levels from 0.0 to 6.0 mg/L in SM under 0.3 mg/L Cu^{2+}, the fraction of SFA, PUFA, and UFA enhanced apparently till 0.3 Cu+2.0 Zn with maximum values of 50.8% SFA, 19.3% PUFA and 22.8% UFA, and then reduced slowly. The proportion of C18:2 and C18:3 also peaked at 0.3 Cu+2.0 Zn, which increased by 30.7% and 97.1% in comparison to $Control_{SM}$, respectively. This implies that an appropriate concentration of Zn^{2+} could alleviate the negative impact of Cu^{2+} on the biosynthesis of specific fatty acids in *S. platensis* biomass.

In reactors with SADE, the diversity of fatty acids in biomass increased with the continuous dilution of SADE (Fig. 3D). In 100% SADE, *S. platensis* cells contained only C16:0, corresponding to an amount of 8.1% of SFA. Fatty acids profile in 50% SADE reactors included C16:0 (28.6%) and C20:3 (18.9%), while C16:0 (31.9%), C18:2 (9.7%) and C18:3 (18.0%) were the main fatty acids in 25% SADE reactors. This indicates that MUFA disappeared in biomass cultivated in SADE reactors. Meanwhile, the amount of C20:3 in 50% SADE reactors improved by 91.4% in comparison with $Control_{SADE}$. Remarkably, a higher dilution ratio was associated with higher fractions of SFA, UFA and PUFA, which increased to 33.8%, 27.7% and 27.7% in 25% SADE reactors, respectively (Fig. 3D). This is likely related to a decrease in the combined toxicity of Cu^{2+} and Zn^{2+} due to the dilution of SADE.

3.3 *Uptake of Cu and Zn in S. platensis biomass*

The uptake capacities of *S. platensis* to different concentrations of Cu^{2+} and Zn^{2+} were determined at the end of the cultivation (Fig. 4). The uptake of Cu^{2+} and Zn^{2+} in *S. platensis* biomass at 0.6 Cu+2.0 Zn was not detected due to the extremely low biomass. In $Control_{SM}$, contents of Cu and Zn were about 24.3 mg/kg dry weight and 29.7 mg/kg dry weight, respectively. Under 0.3 mg/L Cu^{2+} in SM, the uptake of Zn in biomass increased from 87.7 to 1031.7 mg/kg dry weight at 0.0 - 6.0 mg/L Zn^{2+} and then sharply declined to 213.3 mg/kg dry weight at 8.0 mg/L Zn^{2+} ($P < 0.05$) (Fig. 4A). In the absent Zn^{2+} in SM, increase of Cu^{2+} from 0.3 to 0.6 mg/L facilitated the uptake of Cu in biomass from 118.0 to 301.3 mg/kg dry weight ($P < 0.05$). Furthermore, the increase of Zn^{2+} concentration from 0.0 to 4.0 mg/L at 0.3 mg/L Cu^{2+} in SM showed negligible impacts on the uptake of Cu contents (99.0 to 118.0 mg/kg dry weight) but accelerated uptake of Cu in biomass to 386.3 mg/kg dry weight from 4.0 to 8.0 mg/L, indicating

the ability of *S. platensis* to uptake Cu^{2+} was enhanced in the presence of Zn^{2+} > 4.0 mg/L (Fig. 4A). Bioconcentration factor (BCF) is an index to evaluate the ability of microalgae to accumulate heavy metals during the bioconcentration process. A similar trend of BCF of Cu was also observed where BCF remained around 330.0 to 393.3 at Zn^{2+} concentration of 0.0 - 4.0 mg/L but increased continuously reaching 1287.8 at Zn^{2+} concentration of 8.0 mg/L under Cu^{2+} of 0.3 mg/L (Fig. S2). The different initial concentrations of Zn^{2+} tested for *S. platensis* also altered the BCF value of Zn (Fig. S2). Under 0.3 mg/L Cu^{2+} in SM, BCF values of Zn remained 120.7 - 171.9 at 2.0 - 6.0 mg/L Zn^{2+} but declined to 26.7 at 8.0 mg/L Zn^{2+} concentration.

In reactors with SADE, a higher dilution ratio was associated with lower contents of Cu and Zn in biomass (Fig. 4B). The uptake capacities of *S. platensis* biomass significantly decreased from 113.0 in 100% SADE to 27.4 mg/kg dry weight in 25% SADE for Cu and from 1377.3 to 373.4 mg/kg dry weight for Zn with a continuous dilution of SADE ($P < 0.05$).

3.4 Risk assessment

Microalgae could pose health risks through transferring microalgae-associated heavy metals to livestock and humans through food chains when consumed in large amounts at once or in small amounts over a long period of time, even if the concentrations of heavy metals in biomass are low or below toxic levels (Roleda et al., 2019). Thus, the risk of using metal-laden *S. platensis* biomass as a feedstock additive for pigs was investigated.

As seen in Fig. 5A and 5C, a daily consume of 0.075kg of *S. platensis* harvested from the reactors of 2.0 - 4.0 mg/L Zn^{2+} at 0.3 mg/L Cu^{2+} resulted in higher HQ for Cu^{2+}

(0.49 - 0.97) than Zn^{2+} (0.08 - 0.34), despite that ADI values of Zn^{2+} (0.14 - 0.61 mg/(kg·day)) were much higher than Cu^{2+} (0.06 - 0.12 mg/(kg·day)). HI value was the sum of HQ values for Cu and Zn, which was below 1 in low concentration reactors (0.3 mg/L Cu^{2+} and Zn^{2+} ≤ 4.0 mg/L), but greater than 1 in the high concentration reactors (0.3 Cu+6.0 Zn, 0.3 Cu+8.0 Zn, and 0.6 Cu). It is worth noting that ADI values for Cu (0.016 - 0.067 mg/(kg·day)) and Zn (0.22 - 0.81 mg/(kg·day)) of SADE-grown *S. platensis* were all within the permissible limits (0.12 mg/(kg·day) for Cu and 1.80 mg/(kg·day) for Zn (National research Council, 2012)) (Fig. 5B). HI value exceeded 1 in 100% SADE reactors, while HI values in other SADE reactors were much less than 1 (Fig. 5D), indicating *S. platensis* biomass grown in 25% and 50% SADE was acceptable for pig consumption. Furthermore, HQ_{Cu} was higher than HQ_{Zn} in both SM reactors and SADE reactors (Fig. 5C and 5D), although ADI values for Cu were lower than Zn. This indicates HQ_{Cu} is the major contributor to the HI value.

4. Discussion

4.1 Effects of Cu^{2+} and Zn^{2+} on the growth of S. platensis

This study showed that Cu^{2+} (0.3 - 0.6 mg/L) and Zn^{2+} (0.0 - 8.0 mg/L) in SM and SADE system inhibited the production of *S. platensis* biomass. This is likely related to the declined photosynthetic pigment concentrations and increased heavy metals contents in microalgae cells (Fig. 2 and 4). In SM reactors, the presence of Cu^{2+} and Zn^{2+} inhibited the biosynthesis of chlorophyll-a and carotenoid. (Fig. 2A and 2B). The inhibition effect was also positively linked with the Cu^{2+} and Zn^{2+}concentrations. Similarly, reduced chlorophyll-a and carotenoid levels were also observed in SADE with the inherent presence of Cu^{2+} and Zn^{2+} in SADE, where the reduction was alleviated by the dilution of SADE (Fig. 2C and 2D). Decreased chlorophyll-a level in

505 microalgae is a typical sign of metal poisoning (de Filippis et al., 1981). This is likely
506 due to the replacement of magnesium atoms in the chlorophyll porphyrin ring by Cu^{2+}
507 and Zn^{2+} (Kowalewska et al., 1987; Zhou et al., 2018). Cu^{2+} and Zn^{2+} in high
508 concentrations also inhibit the photosynthetic electron transport on the oxidizing side
509 of PS Ⅱand inactivate some PS Ⅱreaction centers (Yang et al., 2015), consequently
510 reducing photosynthesis and inhibiting the growth of microalgae. The result of
511 chlorophyll-a reduction due to Cu^{2+} and Zn^{2+} agreed with the declined chlorophyll-a
512 concentration in *Coelastrella* sp. cells cultivated in ADSW with increased Cu^{2+} levels
513 (0.1 - 3 mg/L) (Li et al., 2018). Carotenoid, acting as an antioxidant, is generally
514 synthesized in large amounts in microalgae cells to protect the cells from oxidative
515 damage due to the enhanced production of reactive oxygen species (ROS) under
516 unfavorable growth conditions such as when being exposed to heavy metals (Foyer and
517 Mullineaux, 1994). In this study, carotenoid production was inhibited due to the toxicity
518 from Cu^{2+} and/or Zn^{2+} in both SM and SADE reactors, where carotenoid concentrations
519 in SM and SADE reactors were significantly lower than their respective control reactors
520 ($P < 0.05$). Similar observation was also found by Kondzior and Butarewicz (2018) that
521 Zn^{2+} in 6.25 - 100 mg/L and Cu^{2+} in 0.025 - 0.15 mg/L in Blue-Green medium (BG 11)
522 both decreased the carotenoids content in *Chlorella vulgaris* cells. Besides carotenoid,
523 Superoxide dismutase (SOD) also acts as the first-line antioxidant enzyme defending
524 against ROS (Saha et al., 2013). A slight increase (7.0%) of carotenoid concentration
525 was observed from day 8 to day 10 in 25% SADE reactors (lowest Cu^{2+} (c.a. 0.17 mg/L)
526 and/or Zn^{2+} (c.a. 0.45 mg/L) concentrations among both SM and SADE reactors) in
527 comparison to $Control_{SADE}$. In contrast, on the 10^{th} day, the activity of SOD, reduced
528 by 12.4% in 25% SADE reactors, but increased by 8.2% and 3.6% in 50% SADE and
529 100% SADE reactors in comparison to $Control_{SADE}$, respectively, (Fig. S2).

Considering the lowest initial cultivation Cu^{2+} and Zn^{2+} condition in 25% SADE reactors and the accumulation of Cu and Zn from the aqueous phase to biomass, it is likely that SOD played the major role in alleviating oxidative stress from ROS when the carotenoid production was inhibited at higher Cu^{2+} and Zn^{2+} concentrations (i.e. 50% and 100% SADE), while the increase of carotenoid production at lower Cu^{2+} and Zn^{2+} condition (i.e. 25% SADE at the later stage of cultivation) reduced the oxidative stress and SOD expression (Gauthier et al., 2020). However, over the whole cultivation, chlorophyll-a, carotenoid and biomass concentrations in SM and SADE reactors with Cu^{2+} and or Zn^{2+} were reduced in comparison to respective control reactors, implying impaired photosynthetic activity of *S. platensis* cells (Fig. 1B, Fig. 2C and 2D). These observations imply toxicity of Cu^{2+} and Zn^{2+} in all SM and SADE reactors potentially had exceeded the total antioxidant production capacity of *S. platensis* in this study.

In SM reactors, increase of Cu^{2+} (0.0 - 0.6 mg/L) and Zn^{2+} (0.0 - 6.0 mg/L) concentration facilitated the uptake of Cu and Zn content in the biomass, respectively. This is consistent with SADE reactors with Cu^{2+} (0.17 - 0.67 mg/L) and Zn^{2+} (0.45 - 1.78 mg/L), where the uptake of Cu and Zn in the biomass was positively related to the Cu and Zn concentrations, respectively. This is likely sufficient active sites existed on the surface of *S. platensis* cells to bind to Cu^{2+} and Zn^{2+}. The higher the concentration of exposed Cu^{2+} and Zn^{2+}, the more Cu^{2+} and Zn^{2+} adsorbed on the cell surface. Moreover, in SM reactors, Zn^{2+} concentration > 4.0 mg/L promoted the uptake of Cu in biomass, indicating the improved selective for Cu^{2+}. This is likely related to the membrane lipid peroxidation and improved membrane permeability resulted from increasing Zn^{2+} level, and thus more substance, such as Cu^{2+}, entered the cell and got internalized. Similar results were reported by Flouty and Estephane (2012) that high levels of Pb^{2+} (> 10^{-6} M) promoted the bioaccumulation of Cu^{2+} by *Chlamydomonas*

556 *reinhardtii* cells, while low levels of Pb^{2+} ($\leq 5\times10^{-7}$ M) had no effect on Cu^{2+}
557 bioaccumulation. This is mainly associated with the presence of two uptake sites of Cu,
558 that is, a Pb-independent, high affinity site and a Pb-dependent, low affinity site (Flouty
559 and Estephane, 2012). However, $Zn^{2+} \geq 8.0$ mg/L at 0.3 mg/L Cu^{2+} inhibited the uptake
560 of Zn, it is likely that the structure of *S. platensis* cells was damaged.
561
562 Cu^{2+} and Zn^{2+} accumulated in cells was found negatively correlated to the biosynthesis
563 of protein (Fig. 3A and 3C). Crude protein production decreased with the increase of
564 Cu^{2+} and Zn^{2+} concentrations in SM reactors (Fig. 3A). Dilution of SADE increased the
565 crude protein level, compared with 100% SADE (Fig. 3B). This suggests that the
566 production of protein was compromised under the presence of Cu^{2+} and Zn^{2+} levels in
567 this study. This is likely due to that Cu^{2+} and Zn^{2+} compete with other metals for the
568 binding sites in proteins following the Irving-Williams series (Mg^{2+} and Ca^{2+} (weakest
569 binding) < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}) (Waldron and Robinson, 2009),
570 leading to the structural destruction, perturbation of protein function and, in some cases,
571 protein degradation (Huertas et al., 2014). Previous studies also showed that production
572 of proteins in *Coelastrella* sp. cells decreased in ADSW with the increased Cu^{2+} content
573 (Li et al., 2018). Alteration of fatty acids caused by Cu^{2+} and Zn^{2+} likely exerted an
574 impact on cell plasma membranes (Wu et al., 2006), subsequently influencing the
575 growth of *S. platensis*. The degree of saturation of fatty acids is associated with the
576 fluidity of membranes and may influence the microalgae response to pollutants (Rocha
577 et al., 2021), i.e., the decrease in PUFA can reduce the fluidity of photosynthetic
578 membranes (Wacker et al., 2016) to result in an alteration in photosynthesis rate
579 (Spijkerman and Wacker, 2011). The fraction of PUFA decreased by 11.4 - 39.5% in
580 25%, 50% and 100% SADE and by 4.8 - 27.2% at 0.3 Cu, 0.3 Cu+6.0 Zn and 0.6 Cu
581 in comparison to their respective control (Fig. 3B and 3D). The growth of *S. platensis*

582 observed in those mentioned reactors was also lower than their respective control (Fig.
583 1A and 1B). A reduction of C18:3 (ω3) amount led to lower incorporation of D1 protein,
584 which is responsible for maintenance of PS II activity (Anderson et al., 1997). C18:3
585 (ω3) disappeared in 50%, 100% SADE and decreased by 20.6% at 0.6 Cu in comparison
586 to $Control_{SM}$. Despite the increase of C18:3 at 25% SADE, 0.3 Cu+2.0 Zn, 0.3 Cu+4.0
587 Zn and 0.3 Cu+6.0 Zn, this was not enough to avoid damage of Cu^{2+} and Zn^{2+} in the
588 photoprotective mechanisms. Therefore, the decline of PUFA, caused by Cu^{2+} and Zn^{2+}
589 in SM and SADE reactors, also weakened photosynthetic performance and hindered
590 microalgal growth of *S. platensis*.
591
592 Furthermore, the growth of *S. platensis* observed on day 11 at 0.6 Cu (GIR: 81.4%, and
593 biomass yield: 0.12 g/L) was comparable to that achieved at 0.3 Cu+6.0 Zn (GIR:
594 85.1%, and biomass yield: 0.09 g/L). Chlorophyll-a (0.67 mg/L at 0.3 Cu+6.0 Zn versus
595 0.52 mg/L at 0.6 Cu) and crude protein (58.4% L at 0.3 Cu+6.0 Zn versus 60.7% 0.6
596 Cu) levels were also comparable between these two reactors, implying *S. platensis*
597 underwent comparable heavy metals toxicity when exposed to 0.3 Cu+6.0 Zn and 0.6
598 Cu. This demonstrates that *S. platensis* was more sensitive to Cu^{2+} than Zn^{2+}. Generally,
599 the changes of metabolites, such as photosynthetic pigments, protein, and fatty acids in
600 cells, can be regarded as an attempt by microalgae to maintain their growth rates or
601 increase their chances of survival under adversity (Li et al., 2018; Rocha et al., 2021;
602 Zhou et al., 2018).
603
604 *4.2 Comparison of growth pattern of S. platensis in different substrates*
605
606 In this study, the growth of *S. platensis* differed between SADE reactors and SM
607 reactors with comparable concentrations of Cu^{2+} and Zn^{2+} (i.e. 100% SADE versus 0.6
608 Cu+2.0 Zn, and 50% SADE versus 0.3 Cu or 0.3 Cu+2.0 Zn). During the cultivation,

609 the biomass concentration of *S. platensis* in 100% SADE (0.63 g/L) was significantly
610 greater than 0.6 Cu+2.0 Zn which was inhibited completely by heavy metals ($P < 0.05$).
611 A similar phenomenon occurred between 50% SADE and 0.3 Cu or 0.3 Cu+2.0 Zn.
612 This is likely that microalgae prefer to utilize the NH_4^+-N in SADE rather than nitrate
613 nitrogen in SM as nitrogen source for biomass accumulation (Cai et al., 2013). Despite
614 the different biomass production patterns between SADE and SM reactors, similar
615 trends of F_v/F_m were observed in the two groups of reactors: (1) 100% SADE and 0.6
616 Cu+2.0 Zn, (2) 50% SADE, and 0.3 Cu or 0.3 Cu+2.0 Zn, implying *S. platensis* cells
617 suffered similarly from combined toxicity of Cu^{2+} and Zn^{2+} in both types of reactors.
618 Chlorophyll-a and carotenoid concentrations in SADE reactors were 73.2% -100% and
619 62.1% - 100% greater than respective SM reactors with comparable Cu^{2+} and Zn^{2+} level,
620 respectively. Chlorophyll-a and carotenoid levels were associated with biomass
621 concentration. Biomass yields of *S. platensis* in SADE were higher than SM reactors
622 with comparable Cu^{2+} and Zn^{2+} level, leading to improved pigments concentrations.
623 SADE is a complex matrix with some substances like tetracycline antibiotics, which
624 may promote the synthesis of pigments (Tong et al., 2020; Zhou et al., 2021). Besides,
625 N element content in SADE is much lower than that in SM reactors (2.50 g/L). N
626 starvation stimulated lipolysis process to produce acetyl-CoA, which was a precursor
627 for synthesis of carotenoid (Kand and Nagarajan, 2013). Crude protein level in 50%
628 and 100% SADE reactors was 61.1% - 64.9% lower than respective SM reactors with
629 comparable Cu^{2+} and Zn^{2+} level. Fatty acids profile of 0.6 Cu+2.0 Zn was not analyzed
630 owing to the low biomass, and no comparison of SFA and UFA between 100% SADE
631 and 0.6 Cu+2.0 Zn was provided in this study. The proportion of SFA and UFA in 50%
632 SADE reactors were 40.9% and 17.1% lower than that at 0.3 Cu+2.0 Zn, respectively.
633 N and P are the major substances for biosynthesis of protein and fatty acids. The

deficiency of N and P in SADE may induce the alteration of metabolic pathways of *S. platensis* and protein might be converted into other biochemical products (Kusmayadi et al., 2022). Besides, different nitrogen sources might be another reason for fatty acids production. The amount of C18:3 at 0.3 Cu+2.0 Zn (8.8%) was significantly greater than 50% SADE (0%). Ronda et al. (2014) also reported NO_3-N is the more suitable for γ-Linolenic acid (C18:3) production of *S. platensis* than NH_4^+-N. Furthermore, it was found that less Cu and more Zn contents were accumulated in 50% SADE-grown biomass than that at 0.3 Cu and 0.3 Cu+2.0 Zn. Although ADI values were both within the allowable range, HI values at 0.3 Cu and 0.3 Cu+2.0 Zn far outclassed 50% SADE. Through experimental implementation and chemical or biological analysis, it was found that growth and biochemical composition of *S. platensis* grown in SADE was differentiated with SM. It is likely influenced by the composition of the culture medium (de souca Oliveira et al., 2021). Considering the potential difference between SM and SADE with comparable Cu^{2+} and Zn^{2+} level, future investigation should focus more on the application in ADE. In addition, previous study identified that bio-adsorption and bioaccumulation are the mechanisms of heavy metal removal by *S. platensis* (Arunakumara et al., 2008; Liliana et al., 2021; Nikokherad et al., 2022), where heavy metals are internalized in cells of *S. platensis* through bioaccumulation. This study aims to evaluate the feasibility of using *S. platensis* biomass cultivated under Cu^{2+}-Zn^{2+} exposure as feed additives for pigs, the detailed mechanisms, and the contributions of bio-adsorption and bioaccumulation for the concentrations of Cu and Zn were not identified, which requires future investigations.

4.3 Implication for S. platensis in the treatment of ADE

Our results showed the promising potential of using ADE as an economical option for

660 the cultivation and harvest of *S. platensis* cells as a feedstock for pig consumption. The
661 economic viability of this system also relies on that *S. platensis* has been proved as an
662 effective supplement in regular animal feeds to improve immune response of animals
663 and enhance meat quality by providing protein and essential fatty acids (Dineshbabu et
664 al., 2019; Zhang et al., 2019). Based on nutritional consideration, the SADE-grown *S.*
665 *platensis* can provide a variety of nutrients needed for pigs and the crude protein (~24%)
666 was equivalent to traditional plant protein sources, such as peas (~22%) and distillers
667 dried grains with solubles (~27%) (Moheimani et al., 2018). The PUFA levels,
668 particularly C18:2 (9.7%) and C18:3 (18.0%) in 25% SADE-grown biomass were
669 comparable with those cultivated in chemicals, thereby providing pigs with abundant
670 PUFA. The risk associated with using the biomass with heavy metal accumulation was
671 assessed in this study. ADI values of Cu and Zn in *S. platensis* biomass grown in SADE
672 systems were all below the limits, and HQ values for Cu and Zn were also less than 1
673 (Fig. 5B and 5D). HI values of diluted SADE (50% or 25% dilution) were also below
674 1, but HI value in 100% SADE was slightly above 1, suggesting biomass from diluted
675 SADE was acceptable for pigs as animal feeds. This is likely related to the high Cu^{2+}
676 (0.67 mg/L) and Zn^{2+} (1.78 mg/L) in 100% SADE, where a consistent higher HI value
677 was observed in SM reactors with $Cu^{2+} \geq 0.3$ and $Zn^{2+} > 4.0$ mg/L. Thus, concentrations
678 of $Cu^{2+} < 0.3$ mg/L and $Zn^{2+} < 4.0$ mg/L are recommended for the application of ADE,
679 where proper dilutions might be essential.
680
681 Based on our laboratory testing results, a comparable amount of biomass could be
682 noticed when *S. platensis* grown in 25% SADE (0.78 g/L) and $Control_{SADE}$ (0.88 g/L)
683 (Fig. 1B). Thus, growing microalgae in diluted SADE yielded comparable biomass with
684 lower chemical use and financial input in comparison to $ControlS_{ADE}$. On the other hand,
685 cultivation in SADE allows for a reduction in harvesting cost owing to the auto-

686 flocculation of biomass. Zeta potential of *S. platensis* cells decreased by 14.2 - 71.3%
687 in diluted SADE reactors and increased by 14.1% in 100% SADE in comparison to
688 $Control_{SADE}$ (Fig. S3). Particles with lower zeta potential values showed a higher
689 tendency for attachment and aggregation (Novoa et al., 2020). Therefore, auto-
690 flocculation of *S. platensis* cells is potentially easier in 25% SADE than 100% SADE
691 and $Control_{SADE}$ due to the lower zeta potential values shown in Fig. S3, making it
692 feasible for reducing the cost of harvesting *S. platensis* biomass. These results indicate
693 that 25% SADE reactors make the reduced costs of biomass production and harvesting
694 technically viable.
695
696 A "circular economy" concept in ADE based on the proposed microalgae technology
697 was established in Fig. 6, relying on the ability of *S. platensis* for nutrient recovery and
698 heavy metals removal in ADE and biomass production used for livestock. In our study,
699 *S. platensis* assimilated 56.2 - 82.9% NH_4^+-N from SADE reactors (Table 3) for
700 biomass production along with the uptake of heavy metals (27.4 - 113.0 mg/kg for Cu^{2+}
701 and 377.4 - 1373.3 mg/kg for Zn^{2+}) (Fig. 4B). This is consistent with the removal
702 efficiency of NH_4^+-N (approximately 38 - 93%) in ADE reported in previous
703 publications (Hasan et al., 2021; Li et al., 2021; Li et al., 2018). The residuals of Cu^{2+}
704 and Zn^{2+} were below than 0.11 and 0.04 mg/L in treated ADE, respectively (Table 3),
705 which was suitable for irrigation (World Health Organization, 2006). The accumulated
706 Cu and Zn in biomass (Fig. 4) indicates *S. platensis* cells can be an effective bio-
707 adsorbent, transferring heavy metals form aqueous solutions to the surface and interior
708 of cells (Chan et al., 2013). These imply that the cultivation of *S. platensis* in ADE can
709 be a potential strategy to remove ammonia and heavy metals from ADE and minimize
710 the pollutions related to the ADE discharge in the environment. The treated ADE can
711 also be recycled for piggery cleaning or water supplement for ADE dilution and

microalgae cultivation to relieve pressure on the water and truly realize water circulation (Fig.6). In addition, this study applied pure species of S. platensis with sterilized laboratory cultivation environments (i.e. culture medium, sterilized ADE, and conical flasks materials) with strict aseptic manipulation to prevent microbial contaminations during the cultivation. In larger-scale setups, potential microbial contaminations might occur during the cultivation, the impact of which requires future investigations.

5. Conclusion

This study explored the physiological and biochemical properties of *S. platensis* cultivated in co-existed Cu^{2+} and Zn^{2+} of SM and SADE reactors. This led to the following findings:

- Presence of Cu^{2+} and Zn^{2+} in SM and SADE resulted in the reduction of biomass, chlorophyll-a and carotenoid concentrations, and production of protein and fatty acids in biomass. The reduction ratio of these compounds was positively related to the Cu^{2+} and Zn^{2+} concentrations.

- *S. platensis* exhibited the ability of nutrient recovery (56.2 - 82.9% of NH_4^+-N) and heavy metals removal in ADE. The excellent uptake capacity reached 99.0 - 386.3 mg/kg in SM and 27.4 - 113.0 mg/kg in SADE for Cu, and 87.7 - 1031.7 mg/kg in SM and 373.4 - 1377.3 mg/kg in SADE for Zn, respectively.

- Using ADE for *S. platensis* cultivation and harvesting the biomass as feedstock additive is promising with economic feasibility (and limited environmental footprint although proper dilution of ADE to Cu < 0.3 mg/L and Zn < 2.0 is essential.

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Table 1 Exposure of *S. platensis* to different concentrations of Cu^{2+} and Zn^{2+} association in SM.

Scenario	Heavy metal treatments		Abbreviations
	Cu^{2+} (mg/L)	Zn^{2+} (mg/L)	
1	0.0	0.0	$Control_{SM}$
2	0.3	0.0	0.3 Cu
3	0.3	2.0	0.3 Cu+2.0 Zn
4	0.3	4.0	0.3 Cu+4.0 Zn
5	0.3	6.0	0.3 Cu+6.0 Zn
6	0.3	8.0	0.3 Cu+8.0 Zn
7	0.6	0.0	0.6 Cu
8	0.6	2.0	0.6 Cu+2.0 Zn

Table 2 Characteristics of the sterilized original anaerobic digestion effluent (SADE).

Parameter	Content (mg/L)
Cu^{2+}	0.67 ± 0.00
Zn^{2+}	1.78 ± 0.04
Mg^{2+}	162.20 ± 0.10
Ammonia nitrogen (NH_4^+-N)	96.15 ± 0.45
Chemical oxygen demand (COD)	407.80 ± 0.00

Table 3 Residual of Cu^{2+} and Zn^{2+}, and removal of NH_4^+-N by *S. platensis* in SADE. Data are presented as mean values ± standard deviations in biological duplicates (n = 3).

Dilution	Heavy metal (mg/L)		NH_4^+-N (mg/L)		
	Cu	Zn	Day 0	Day 10	Removal efficiency (%)
100%	0.11 ± 0.01	0.038 ± 0.00	96.2 ± 0.5	15.9 ± 1.1	82.9 ± 0.9
50%	0.068 ± 0.00	0.017 ± 0.00	47.8 ± 1.5	18.3 ± 1.2	61.8 ± 1.2
25%	0.028 ± 0.00	0.013 ± 0.00	23.6 ± 2.9	10.5 ± 2.6	56.2 ± 5.2

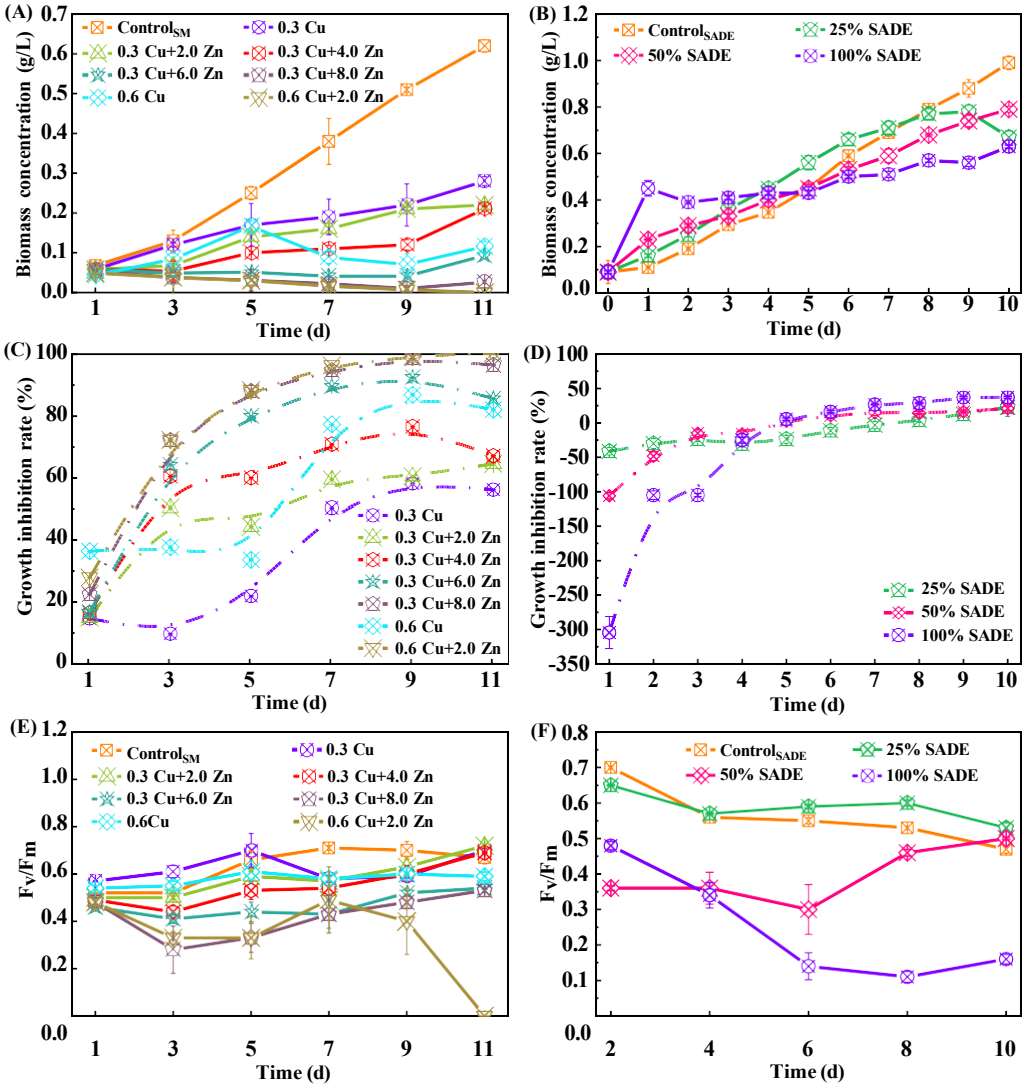

Fig. 1 Growth performance of *S. platensis* in different reactors during the cultivation. (A), (C) and (E) were the biomass concentrations, growth inhibition rate (GIR) and F_v/F_m (the maximum photochemical quantum yield of Photosystem II) of *S. platensis* cells grown in SM with different concentrations of Cu^{2+}-Zn^{2+} association, respectively. (B), (D) and (F) were the biomass concentrations, GIR and F_v/F_m of *S. platensis* biomass cultivated in reactors with different dilution of SADE, respectively. Data are presented as mean values ± standard deviations in biological duplicates (n = 3).

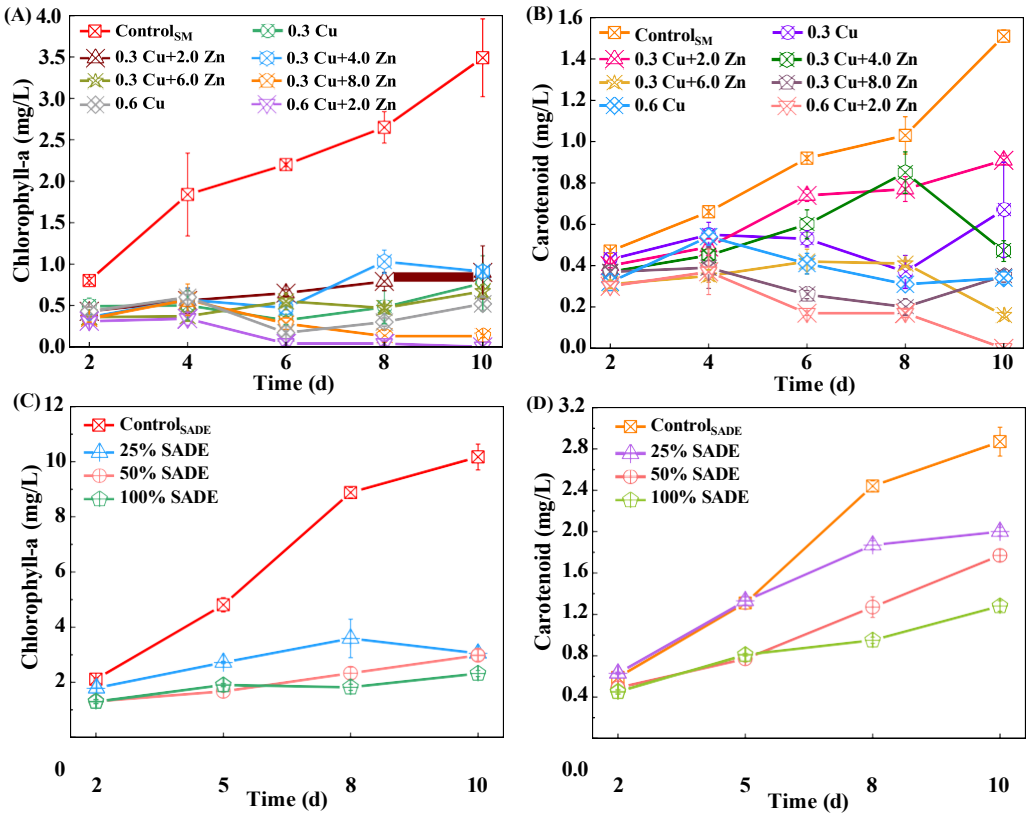

Fig.2 Concentrations of photosynthetic pigments in *S. platensis* biomass when exposed to different reactors. (A) and (B) were chlorophyll-a and carotenoid in reactors with SM and different concentrations of Cu^{2+}-Zn^{2+} association, respectively. (C) and (D) were chlorophyll-a and carotenoid in reactors with different proportions of SADE. Data are presented as mean values ± standard deviations in biological duplicates (n = 3).

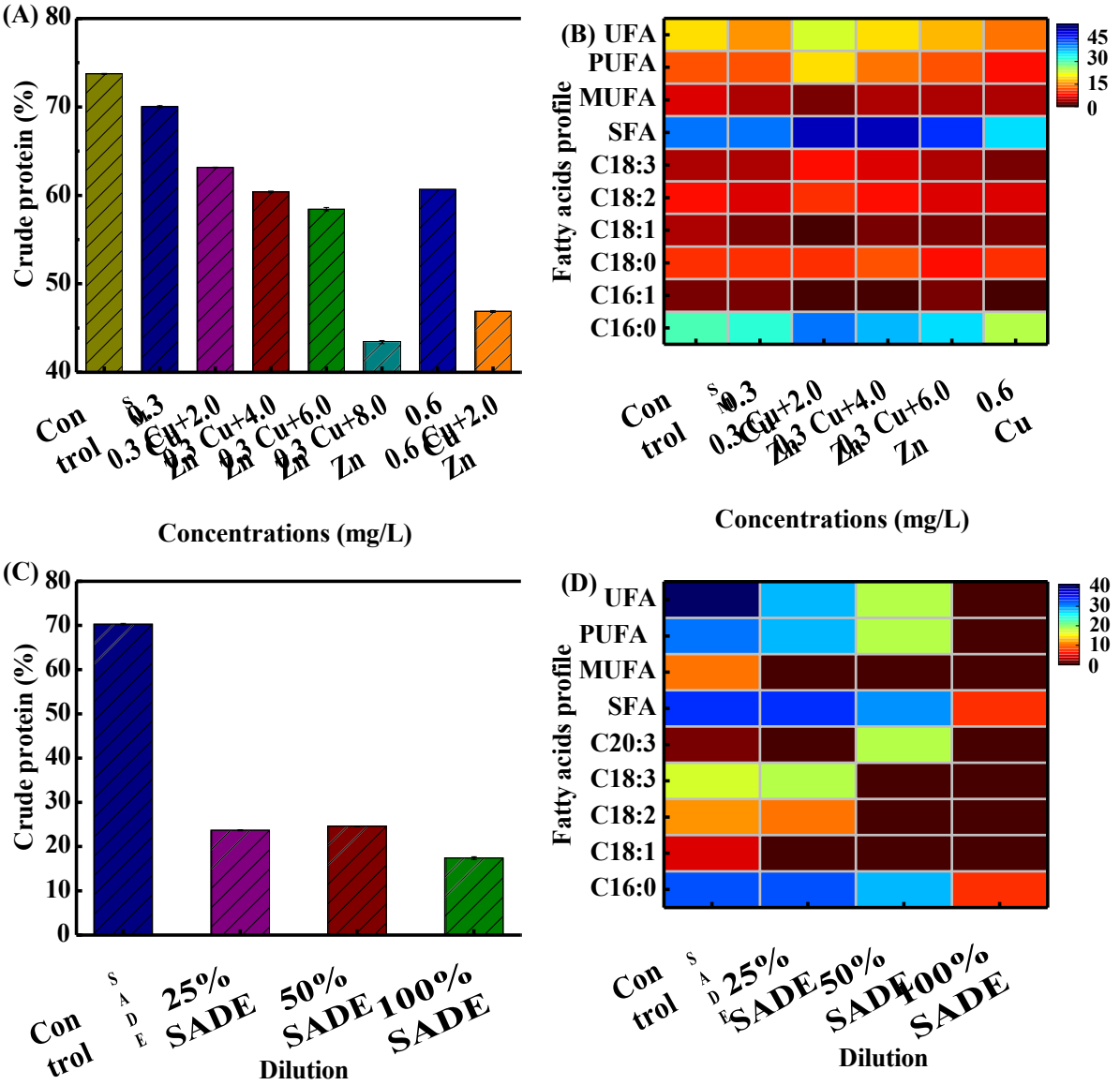

Fig. 3 Crude protein contents in Cu^{2+}-Zn^{2+} associated SM reactors (A) and sterilized anaerobic digestion effluent (SADE) reactors (C); fatty acids profiles in Cu^{2+}-Zn^{2+} associated SM reactors (B) and SADE reactors (D). Data were measured at the end of the cultivation. Error bars represent standard deviations (n = 3).

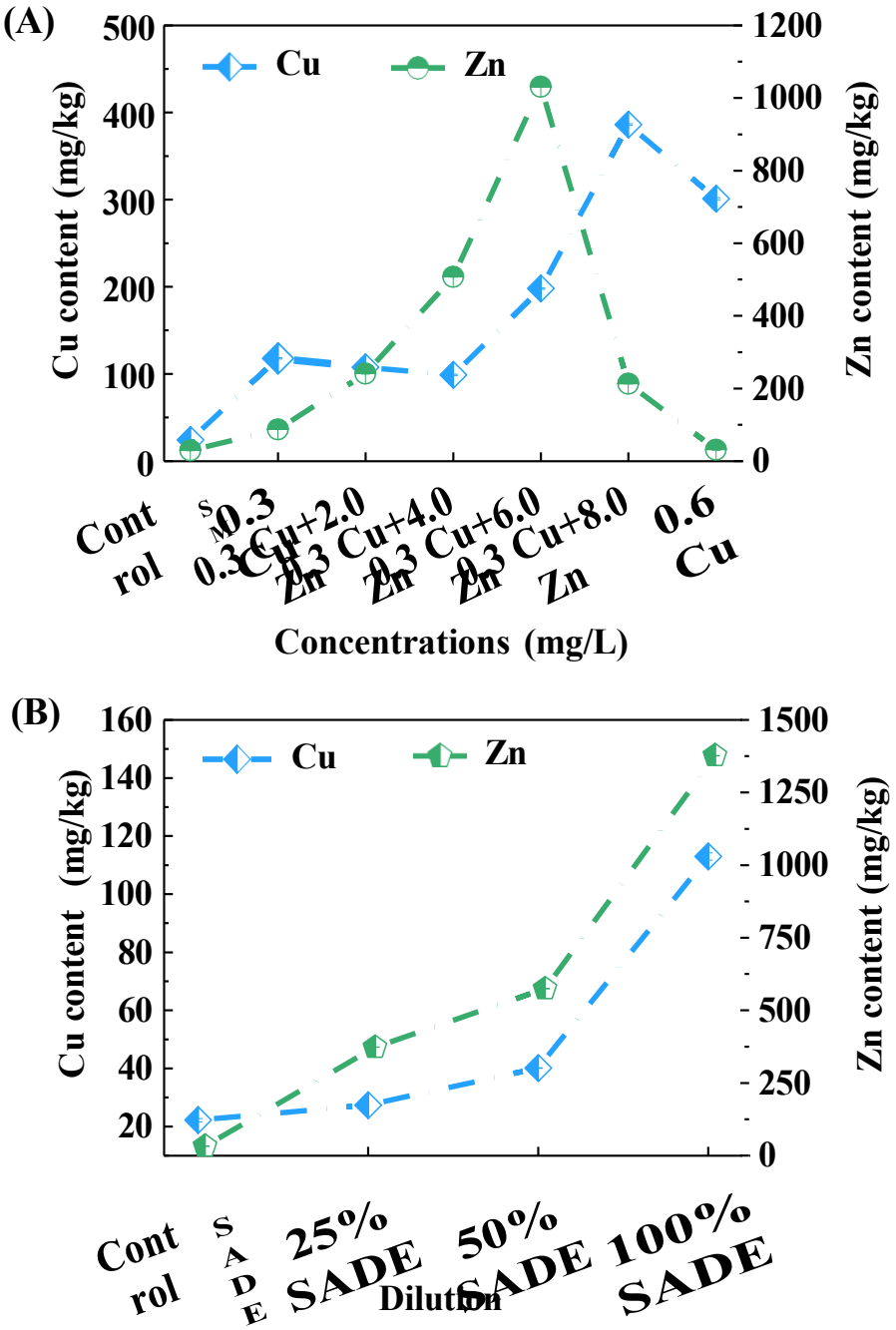

Fig. 4 Uptake of Cu^{2+} and Zn^{2+} in *S. platensis* biomass in Cu^{2+}-Zn^{2+} associated SM reactors (A) and sterilized anaerobic digestion effluent (SADE) reactors (B) (with standard deviations (n = 3)).

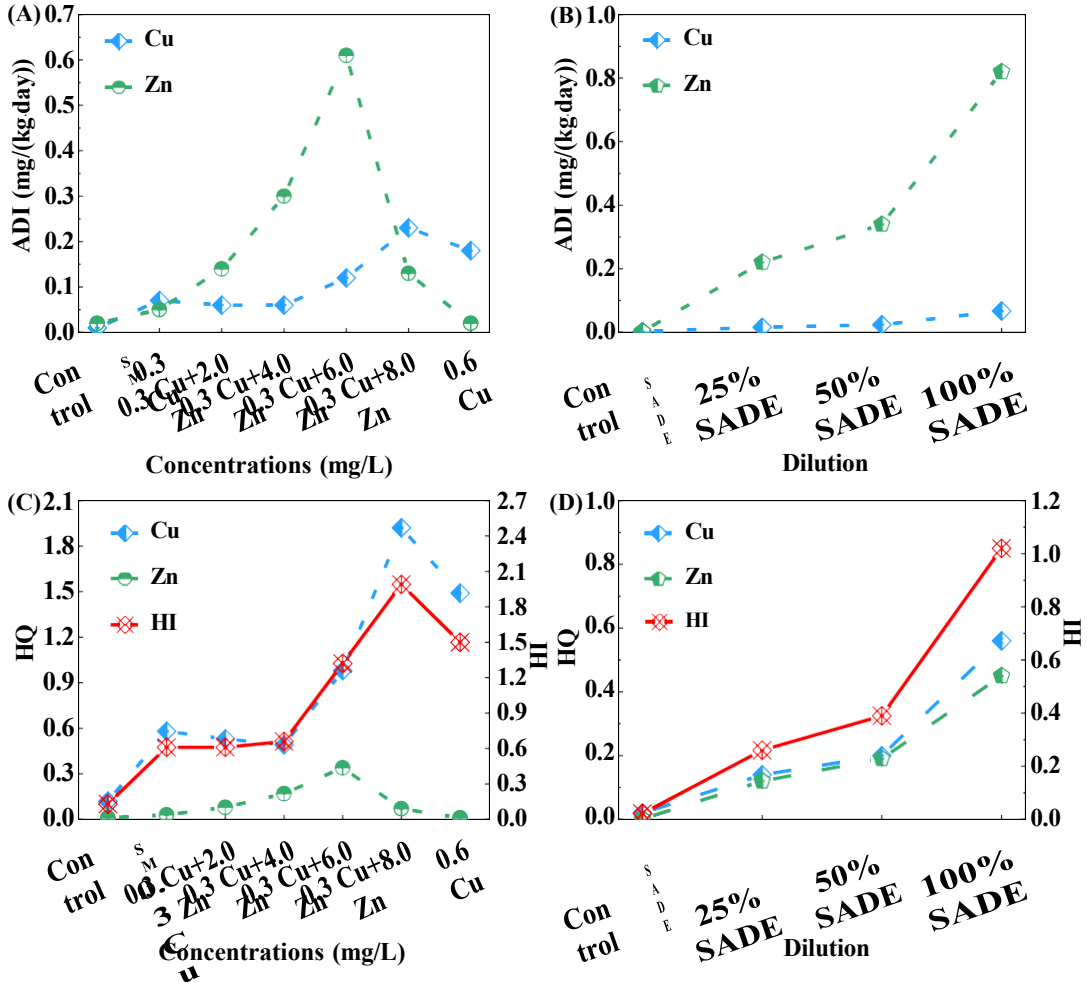

Fig. 5 Risk assessment of harvested *S. platensis* biomass digested by pigs. (A) and (B) mean ADI (average daily intake) of Cu and Zn from *S. platensis* grown in Cu^{2+}-Zn^{2+} associated SM reactors and sterilized anaerobic digestion effluent (SADE) reactors, respectively. (C) and (D) represent the hazard quotient when pigs were fed on *S. platensis* biomass cultivated in Cu^{2+}-Zn^{2+} associated SM reactors and SADE reactors (with standard deviations (n = 3)).

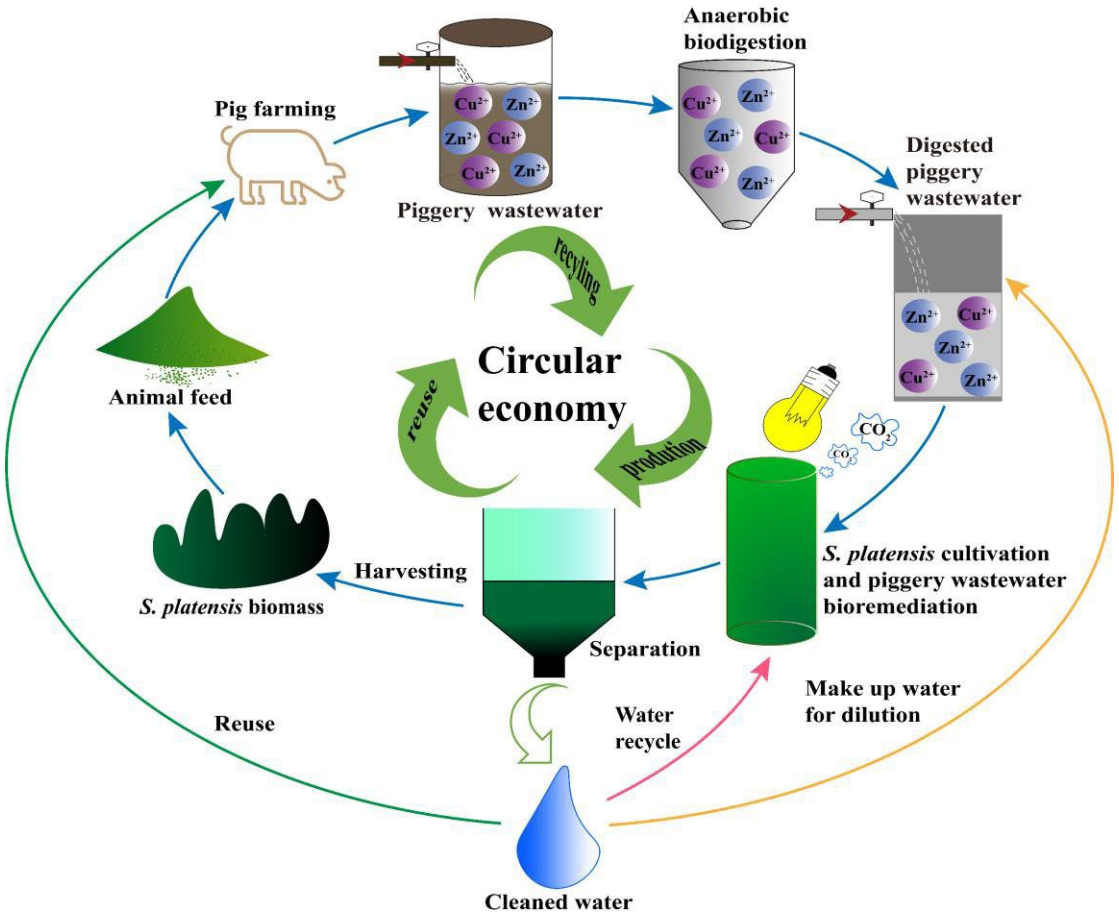

Fig. 6 Schematic representation of anaerobic digestion purification and sustainable utilization of *S. platensis* biomass in the circular economy framework.