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Effect of calcium peroxide pretreatment on the remediation of sulfonamide antibiotics (SMs) by *Chlorella* sp.

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Abstract

This study investigated the effect of CaO_2 pretreatment on sulfonamide antibiotics (SMs) remediation by *Chlorella* sp. Results showed that a CaO_2 dose ranging from 0.05 to 0.1 g/g biomass was the best and led to higher SMs removal efficacy 5-10% higher than the control. The contributions made by cometabolism and CaO_2 in SMs remediation were very similar. Bioassimilation could remove 24% of sulfadiazine (SDZ) and sulfamethazine (SMZ), and accounted for 38% of sulfamethoxazole (SMX) remediation. Pretreatment by CaO_2 wielded a positive effect on microalgae. The extracellular polymeric substances (EPS) level of the CaO_2 pretreatment microalgae was three times higher when subjected to non-pretreatment. For the long-term, pretreatment microalgae removed SMs 10–20% more than the non-pretreatment microalgae. Protein fractions of EPS in continuous operation produced up to 90 mg/L for cometabolism. For bioassimilation, SMX intensity of the pretreatment samples was 160-fold less than the non-treatment one. It indicated the CaO_2 pretreatment has enhanced the biochemical function of the intracellular environment of microalgae. Peroxidase enzyme involved positively in the cometabolism and degradation of SMs to several metabolites including ring cleavage, hydroxylation and pterin-related conjugation.

Keywords: Calcium peroxide, cometabolism, microalgae, sulfonamide, extracellular polymeric substances, peroxidase enzyme

1. Introduction

Sulfonamide antibiotics (SMs) are common medications for bactericidal in humans and animals. SMs group includes some typical compounds such as sulfamethoxazole (SMX), sulfadiazine (SDZ), and sulfamethazine (SMZ) (Cheng et al., 2020). Demand for SMs in the marketplace has become more widespread in recent years, leading to rising traces of SMs concentrations in the environment (Luo et al., 2014). For example, SMX was detected at a concentration of 0.98 – 1.1 μg/L in water sources in several countries such as the USA, France, Spain, the United Kingdom, and South Korea (Luo et al., 2014). They are detected in both terrestrial and aquatic environments (Zhou et al., 2019). Long-term exposure to SMs can result in antibiotic-resistant bacteria and genes (Cheng et al., 2020). Those pathogens are eventually transmitted to people, and it poses a great threat to public health.

Various technologies have been developed to deal with the trace amount of SMs in water sources (Luo et al., 2014). Microalgae technology is a particular bioprocess for pollutants' remediation. In water sources, SMs exist at trace concentration ranging from nano to micrograms per liter. This amount cannot provide a sufficient carbon source for microalgae because they need to consume other carbons such as glucose, carbonate which present at milligram per litre in wastewater. Simultaneously, microalgae extrude extracellular polymeric substances (EPS) and several types of enzymes to cometabolize micropollutants (Vo et al., 2020b). For this reason, cometabolism is a feasible option to remediate SMs. Bioassimilation is also an important part of the whole biodegradation process. Some studies have investigated micropollutants remediation focusing on bioassimilation, but still lack details on the antioxidant enzymes involved and transformation pathways of micropollutants' degradation products (Chen et al., 2017; Xiong et al., 2020). Our understanding of cometabolism and bioassimilation in microalgae towards SMs remediation is still in its nascent stages. In our previous study, we investigated the effects of various carbon sources on micropollutants' cometabolism of microalgae (Vo et al., 2020b). The most efficient one is glucose, which helps microalgae produce the highest EPS and enzymes. However, the issue is that sulfamethoxazole is cometabolized only in minimal amounts (25-50%), a problem that is tackled here. We conducted a novel method to increase SMs remediation by microalgae.

CaO_2 is a crystalline peroxide which is considered a solid form of hydrogen peroxide (H_2O_2). When exposed to aqueous media, CaO_2 degrades and forms alkaline, $•OH^-$, $•O_2^-$, and

Ca^{2+}. CaO_2 is used commercially for several applications such as a bactericide, preservative, medicine, energy recovery and environmental remediation (Wang et al., 2019a; Zhang et al., 2020). For example, CaO_2 is applied to the pretreatment of waste activated sludge for hydrogen production (Wang et al., 2019b). The underlying mechanism of how CaO_2 pretreatment impacts on microorganisms is not clearly understood.

Up to the best of our knowledge, there have not any existed reports using CaO_2 for pretreatment of microalgae towards micropollutants remediation. We hypothesized that CaO_2 pretreatment increases SMs cometabolism and bioassimilation of microalgae. Our hypothesis is based on the results of the plant science studies (de Azevedo Neto et al., 2005; Ozaki et al., 2009). Peroxide pretreatment brings substantial value to the intracellular systems of cells. For example, the photosynthesis, chloroplastic and cytosolic activities rose (Ozaki et al., 2009). By using 1 μM peroxide for pretreatment lasting 2 d, the activity of lipid peroxidation and antioxidative enzymes (e.g., superoxide dismutase, ascorbate peroxidase, and guaiacol peroxidase) increased by 70% (de Azevedo Neto et al., 2005). The increase in antioxidant enzymes alleviates the environmental stresses of drought, salinity, pollutants (e.g., heavy metals, organic pollutants) to plants. Microorganisms also extrude extra organic matter and EPS to form soluble EPS while applying 25 – 35 mg/g VSS of peroxide pretreatment (Liang et al., 2020). All those EPS and antioxidative enzymes potentially contribute to SMs cometabolism and bioassimilation. Therefore, to unveil the hypothesis, several questions need to be elaborated. Does CaO_2 degrade SMs by itself or support cometabolism and bioassimilation, and how much does each factor contribute? Does CaO_2 pretreatment stimulate microorganisms extruding more EPS and oxidative enzymes for cometabolism and bioassimilation? What are the major and minor degradation products of SMs using CaO_2 pretreatment?

In this study, we investigated the effect of CaO_2 pretreatment for SMs remediation by microalgae, focusing on cometabolism and bioassimilation. The objectives are to: (i) assess an optimal CaO_2 dosage (0–0.25 g/g biomass) for pretreatment; (ii) quantify the contribution of CaO_2, cometabolism, and bioassimilation to SMs remediation; (iii) evaluate SMs remediation in a long-term scenario; (iv) study the production of EPS and antioxidant enzymes for cometabolism and bioassimilation; and (v) unveil the mechanism of CaO_2 pretreament on SMs degradation and transformation pathways. This work explains the underlying mechanism of how CaO_2 pretreatment increased SMs remediation with important insights into the biochemical functions and transformation pathways of SMs. CaO_2 pretreatment for micropollutants remediation by microalgae demonstrates much potential for upscaling purposes.

2. Materials and methods

2.1 Microalgae strain, artificial wastewater, and chemicals

Microalgae strain (*Chlorella* sp. CS-436) was purchased from the National Algae Supply Service (Tasmania, Australia). This strain is selected based on the results of previous works that *Chlorella* sp. CS-436 performed efficient pollutants remediation and enzyme generation (Vo et al., 2019; Vo et al., 2020a). The stock of microalgae was incubated in MLA media, which was supplied by AusAqua (South Australia, Australia). The temperature was maintained at 20±1 ^{o}C. Illumination was supplied 24h per day given at 4.35 ± 0.03 klux. The illumination intensity was monitored by a digital light meter, model QM1584 (Digitech, Australia). Microalgae stock was freshly prepared every two weeks.

Artificial wastewater, prepared with RO water, was used for the experiments. The nutrients for microalgae included $C_6H_{12}O_6$, $NaNO_3$ and KH_2PO_4 with concentrations of 300, 30 and 5 mg/L, respectively. Trace vitamin was purchased from AusAqua Pty Ltd (South Australia, Australia). The dose of trace vitamin was advised by the guideline of the supplying

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company, i.e. 1 ml per 1 m^3 medium. The initial pH and dissolved oxygen (DO) of the artificial wastewater were 6.9 and 8.2 mg/L, respectively. The experimented SMs included SDZ, SMX, and SMZ. Those micropollutants (MPs) were stocked in advance at 1 g/L and spiked to reactors at 200 μg/L. The details of the properties of these MPs are summarized in Table S1. All chemicals were supplied by Merck (New South Wales, Australia) at analytical grade quality.

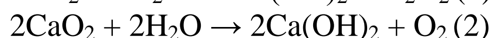
2.2 Experimental designs

2.2.1 First batch study to determine the optimal CaO_2 dose

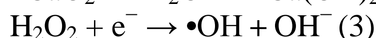
Glass photobioreactors of 1 L volume were used for the first batch experiment. Those reactors were sealed for the whole experiment. 10% of the reactors' volume consisted of air that remained inside for microalgae respiration. The culturing liquid of the reactor was mixed by a magnetic mixer at a steady rate (50 rpm) to increase interactions of microalgae and nutrients, illumination, MPs, and CaO_2. The seeding concentration of microalgae in the reactor was 50 mg/L. Six sets of photobioreactors were prepared in which were dosed with CaO_2 at 0, 0.05, 0.1, 0.15, 0.2 and 0.25 g/ g biomass. This set of experiment was warranted by duplication.

All photobioreactors were operated with CaO_2 and without MPs over 2 d for adaptation. After the pretreatment stage, extracellular polymeric substances (EPS), enzyme and chlorophyll were measured as described in sections 2.3 - 2.5. The concentrations of Ca^{2+}, $•OH^-$ and $•O^{2-}$ were calculated by using the oxygen releasing rate of Zhou et al. (2017) as shown in Eq (1) – Eq (5):

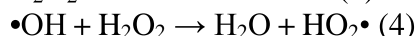
$CaO_2 + 2H_2O \rightarrow Ca(OH)_2 + H_2O_2$ (1)



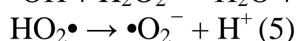
$2CaO_2 + 2H_2O \rightarrow 2Ca(OH)_2 + O_2$ (2)



$H_2O_2 + e^- \rightarrow •OH + OH^-$ (3)



$•OH + H_2O_2 \rightarrow H_2O + HO_2•$ (4)



$HO_2• \rightarrow •O_2^- + H^+$ (5)

The calculation showed that CaO_2 was dissolved completely in the pretreatment stage and its residues were generated (section 3.2). MPs were also dosed in the respective photobioreactors (200 μg/L for each MP) as described. The first batch experiment lasted 10 d. The pH in all the reactors was kept at 6.9 ± 0.2 thoroughly by sulphuric acid or sodium hydroxide after the pretreatment stage.

2.2.2 Second batch study to differentiate contributions of CaO_2, cometabolism, and bioassimilation in MPs degradation

The second batch study was carried out to quantify the contribution of CaO_2, cometabolism, and bioassimilation in SMs degradation. This experiment was conducted in three identical Erlenmeyer flasks with duplication. The three flasks contained: (i) extracted liquid, (ii) CaO_2 and extracted liquid, and (iii) only CaO_2 in mili-Q water, respectively. The extracted liquid contained EPS and oxidative enzymes, which were extruded by microalgae. The extracted liquid was obtained by filtering the culturing liquid of microalgae by 1.2 μm Phenex-GF (Glass fiber). Microalgae were cultured beforehand by using the same procedure of the first batch experiment. The CaO_2 dose, which was applied in the second batch study, was 0.1 g/ g biomass which was optimized from the first batch study. Three MPs were dosed at 200 μg/L for each compound similarly to the first batch experiment. The second batch experiment lasted 10 d with sampling occurring every 2 d. The pH was also maintained at 6.9 in this experiment.

2.2.3 Long-term study to assess the impact of CaO_2 pretreatment

The long-term study was designed in semi-continuous mode to assess the significance of CaO_2 pretreatment. Two photobioreactors were set up and monitored over a 60 d period. One reactor was dosed with CaO_2 (0.1 g/ g biomass) and one without CaO_2. Sampling was conducted every 5 d via three steps. The mixers were turned off to settle biomass in 30 min. 900 ml of the supernatant was withdrawn for sampling and 100 ml of the settled biomass

remained. After sampling, 900 ml of DI water was refilled in each reactor. SMs, CaO_2 and nutrients were re-dosed, and the subsequent concentrations were similar to the batch studies. On days 15 and 35, 20 ml of biomass (20% of total biomass volume) was withdrawn to condition the new biomass so that it could grow.

2.3 Biomass concentration and chlorophyll

Methods to calculate and analyze biomass concentration and chlorophyll were mentioned in a recent work (Vo et al., 2020b). To determine biomass concentration, the sample was measured for optical density (680 nm) by spectrophotometer (DR1900, Hach). The optical density was converted to a biomass concentration by Eq. 6 (Vo et al., 2019), which is written below as follows:

$$y = 0.0021x + 0.0222 \ (R^2 = 0.95) \quad (6)$$

Where, x represents concentration of biomass (mg/L), y represents optical density

Chlorophyll *a* was analyzed by the following steps. A sample (10 ml) was centrifuged at 10,000 rpm in 10 min. The supernatant was disposed, and pellet was incubated in 10 mL acetone (90% v/v) at 4 ^oC in 24 h in a dark room. After incubation, the mixture was centrifuged at 4 ^oC, 4000 rpm in 15 min. The supernatant was scanned by a spectrophotometer at four wavelengths: 750 nm, 664 nm, 647 nm, and 630 nm. Acetone (90% v/v) served for the blank sample. Chlorophyll *a* concentration was calculated as Eq. 7 below:

$$\text{Chlorophyll } a \text{ (mg/L)} = 11.64*(OD_{663}-OD_{750}) - 2.16*(OD_{647}-OD_{750}) + 0.1*(OD_{630}-OD_{750}) \quad (7)$$

Where, OD_λ: optical density at wavelength λ (nm).

2.4 Extraction and analysis of extracellular polymeric substances

The EPS were extracted and analysed using the methods employed by Deng et al. (2016) and Ni et al. (2009). One sample (30 mL) was centrifuged at 3000 rpm for 30 min and filtered by a 0.45 µm Phenex-NY (Nylon) syringe to achieve soluble EPS. The pellet was incubated in 30 mL phosphorus buffer solution with cation exchange resin (Dowex) for 2 h at 900 rpm. After filtering the mixture through a 1.2 µm Phenex-GF (Glass fiber) syringe, bound EPS was attained. The soluble and bound EPS were analyzed for proteins and polysaccharides. The Lowry method was used for protein analysis and the Anthrone-sulphuric acid method was applied for carbohydrate analysis. The Lowry method was based on the modified Lowry kit (Sigma, Australia). This method uses sodium dodecylsulfate and the Lowry reagent for the dissolution of relatively insoluble lipoproteins. Protein concentration (absorbance at 750 nm) is determined from a standard curve.

2.5 Extraction and analysis of peroxidase enzymes

The reactivity of peroxidase (POX) enzyme was analyzed by the POX assay kit (Sigma-Aldrich, Australia). The sample (20 ml) was centrifuged at 4500 rpm for 15 min at 4 ^oC. The received supernatant was used to measure the reactivity of POX in the extracellular environment of microalgae. The pellet was washed by Milli-Q water and refilled to 20 ml. The pellet sample was frozen at -20 ^oC and then thawed at room temperature (37 ^oC). After thawing, the sample was sonicated for 1 h and centrifuged for 10 min at 4500 rpm. The received supernatant was for measuring POX activity in the intracellular cells of microalgae. The POX activity was undertaken following the manufacturer's instructions. The unit U/ml (nmol/min.ml) was used to demonstrate POX reactivity.

2.6 Micropollutants analysis

Samples (50 ml) were collected in the fume hood to minimize the effect of external conditions, spiked internal standard (Tetracycline – 2 µg/L), and shaken vigorously to remove the MPs adsorbed onto the microalgae's surface. The supernatant measured the residual MPs in the culturing liquid. The analysis was performed by liquid chromatography equipped with mass spectrometry (LC-MS 8060 Shimadzu), assisted by a Luna Omega 3 µm Polar C18 column (Phenomenex, Australia). The MS was operated by an electrospray ionization (ESI) source (Thermo Fisher Scientific, USA). All the SMs and tetracycline were performed by the

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ESI positive mode. Specifically, the mobile A phase included Milli-Q water with 0.1% formic acid, while the mobile phase B was methanol. The gradient elution was adjusted at a fixed flow rate (0.4 ml/min). The mobile phase B was held at 30% (0.01 to 0.29 min), rose to 95% (0.29 to 7 min), and fell to 30% (7 to 7.5 min). The multiple reaction modes (MRMs) of SMX (254.1>156, 254.1>92), SMZ (279.1>186, 279.1>92.1), SDZ (251.1>156.1, 251.1.1>92.1) and TC (445.25>410, 445.25>428) were selected.

2.7 Metabolites analysis

The collected samples were centrifuged at 4500 x g for 15 min. The supernatant was used for detecting the degradation products of SMs in the culturing liquid. The cell pellet was washed with Milli-Q water. The washed pellet was sonicated (350W, 50 Hz) for 1 h to promote cell lysis and centrifuged for 10 min at 4500 x g. Then the supernatant was concentrated by solid phase extraction (SPE) using Strata-X-C 33 µm, Polymeric Strong Cation (Phenomenex, Australia). The SPE cartridge was eluted with 10 ml methanol, condensed to 1 ml via a gentle nitrogen stream. The eluant was collected for analyzing the degradation products in microalgae cells.

The samples were examined on a HPLC-TOF-MS system consisting of an Agilent 1200 HPLC (Agilent Technologies, CA, U.S.) linked to a Bruker Daltonics micro-TOFq mass spectrometer (Bruker Daltonics, Bremen, Germany). Analytes were separated using a CORTECS T3 column (2.7µm, 100 x 3 mm, Waters Corp., Milford, U.S.A). The mobile phases were 0.1% formic acid in water (A) and acetonitrile (B). The flow rate was 0.3 mL/min and the oven temperature was 35 °C. The employed gradient elution was: isocratic at 5% B for 3 min rose to 95% B over 15 min and kept constant for 8 min. The initial conditions were returned in 1 min and then equilibrated for a total run time of 30 min. All samples were analysed in both ESI positive and negative mode. The received data were compared with the local library which included potential metabolites and degradation products in the literature using TASQ 2.1 software (Bruker Daltonics, Bremen, Germany). The mass accuracy for a positive identification was set at 5 ppm.

2.9 Contributions of the degradation factors in SMs remediation

Based on the mass balance, we established the following equation to quantify the contributions of the degradation factors to SMs remediation:

$$C_c + C_{CaO2} + C_r + C_a + C_b = 100\% \quad (3)$$

Where:

C_c (%): the percentage of SMs remediated by the cometabolism (%), scavenged by EPS.

C_{CaO2} (%): the percentage of SMs remediated by CaO_2 (%)

C_r (%): the percentage of SMs in the aqueous phase. C_r comprised of the remaining SMs in the aqueous phase at the end of the experiments and adsorbed on microalgae cells' surface.

C_c, C_{CaO2} and C_r were obtained from the second batch experiments.

C_a (%): percentage of SMs degraded by the abiotic control. This fraction was negligible (0 - 2.1%) from literature (Xiong et al., 2019; Xiong et al., 2020)

C_b (%): the percentage of SMs remediated by bioassimilation. C_b was calculated using Eq (6) where other components were known.

2.10 Statistical analysis

Analyses of variance (ANOVA) were used for statistical investigations. Repeated ANOVA measures examined the significant differences in MPs removal efficiencies. The removal efficiencies of MPs, EPS level, reactivity of enzymes and chlorophyll were measured in duplicate. Statistical analyses were calculated by Origin 2017 (Origin Lab, USA) software and any such emerging statistical differences were based on the values of means with a 95% confidence level.

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3. Results and discussion

3.1 Assess the optimal CaO_2 doses for the pretreatment

To evaluate the optimal doses of CaO_2, 5 doses (0-0.25 g/g biomass) were tested and the concentrations of SMX, SDZ, and SMZ were measured at day 10 (Fig. 1a). The results showed that the optimal CaO_2 doses ranged from 0.05 to 0.1 g/g biomass, which could remove up to 60% of the spiked SMs. Those removal efficacies were higher than the non-pretreatment sample, i.e. 5 to 10%. The pretreatment using 0.1 g CaO_2/g biomass has brough many benefits in chlorophyll and EPS generation, and assisted microalgae to degrade SMs in a greater extent (section 3.4 – 3.7). The CaO_2 doses, which exceeded 0.1 g/g biomass, did not perform significant removal at day 10. The optimal CaO_2 doses (0.05 – 0.1 g/g biomass) could eliminate SMs up to as much as 130 μg (Fig. 1b). A dose of 0.1 g/g biomass was selected for the subsequent experiments. This dose was in the optimal range of 0 – 0.14 g/g biomass, which has been investigated previously (Wang et al., 2019a; Wang et al., 2018a). We observed a sudden drop in SMs removal efficiencies when CaO_2 dose was greater than 0.15 g/g biomass. This phenomenon potentially attributed to the negative effect of excessive free radicals to *Chlorella* sp. activity in the reactors. Further explains regarding the effects of CaO_2 residues are provided in section 3.2.

[Insert Fig. 1]

3.2 How CaO_2 residues affect microalgae?

CaO_2 caused critical effects to the SMs remediation system by the slow-releasing oxygen characteristic. Upon spiking into the reactors, CaO_2 reacted with H_2O and formed alkaline, Ca^{2+}, $•O_2^-$ and $•OH^-$and changed the water chemistry such as pH and DO (Ali et al., 2020; Wang et al., 2019b). Those residues of CaO_2 posed certain changes to the biological behaviour of microorganism. For example, CaO_2 altered the acidogenesis, acetogenesis and methanogenesis processes of the dark fermentation processes (Wang et al., 2019a). In this study, CaO_2 influenced on the intracellular and extracellular enzyme, chlorophyll and EPS extrusion of microalgae which determined the SMs remediation. Amongst 4 residues, alkaline and Ca^{2+} did not show any potential impacts because pH was controlled at 6.9 in the experiment. We calculated the concentrations of other residuals by using the referring oxygen-releasing rates of Zhou et al. (2017) (1.5 to 4.2 mg/L.h.g CaO_2). The calculated concentrations of Ca^{2+}, $•O_2^-$ and $•OH^-$ after 48 h pretreatment were 4.1 - 33.4 mg/L, 1 - 8.3 mg/L and 2 – 16.7 mg/L, respectively. The dosing CaO_2 in the reactors was all dissolved after pretreatment. The calculations agreed to Wang et al. (2019a) and Wang et al. (2019b) who found that the residues were generated substantially after 48h of the pretreatment stage.

As previously mentioned, Ca^{2+} did not cause negative impacts to the microalgae because it was a vital element for the biochemical functions. For example, exogenous Ca^{2+} concentrations from 6.8 to 400 mg/L alleviated the detrimental effects of radical groups to microalgae (Husseini et al., 2020). The Ca^{2+} played role as an additional substrate to increase growth rate, catalase activity, proline, and carotenoid content and peroxidase enzyme. The Ca^{2+} ion was an important part of EPS and biofilm chemical structures (Urbain et al., 1993). The exogenous Ca^{2+} decreased membrane damages by up-regulating the activities of CIPK/CBL genes, glutathione, calmodulin and *MAPK*, resulting in the increase of biomass and lipid accumulation (Hirschi, 2004; Qiao et al., 2020). To validate the effects of Ca^{2+}, Wang et al. (2019a) used the alternative substrate ($CaCl_2$) at the similar CaO_2 doses of this study and confirmed that Ca^{2+} improved the methane production of the waste activated sludge digestion. Therefore, the influences of $•O_2^-$ and $•OH^-$ to microalgae were significant and taken into account rather than Ca^{2+}. By using H_2O_2 for pretreatment (17 – 136 mg/L), the cell growth rates of microalgae decreased by 3.9 – 35.4% and, in contrast, the protein content increased by 32.8% (Qiao et al., 2020). The results of Qiao et al. (2020) agreed to our findings referring to biomass diminution (50 – 75%) and EPS increased (300%) due to the stress of $•O_2^-$ and $•OH^-$ species (Fig. 3 and Fig. S1). The Reactive oxygen species (ROS) groups have

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been known of causing cells damage (Ugya et al., 2020). They encountered the cell membrane and molecules such as DNA. To reduce the stress of ROS, microalgae produced antioxidant species such as chlorophyll and peroxidase enzyme to eradicate the radical species to H_2O molecules (Griveau and Lannou, 1997). Consequently, the SMs removal efficacies were reduced by increasing CaO_2 doses.

3.3 Evaluate the contribution of CaO_2, cometabolism, and bioassimilation in SMs remediation

The solid CaO_2 possessed a strong oxidative state and could mineralize SMs at a certain level. Our calculation showed that when spiking CaO_2 into the reactors for pretreatment, the hydroxyl radical groups remained in the reactors and oxidized SMs together with EPS and enzymes (section 3.2). The first batch experiment in section 3.1 showed that 0.1 g/g biomass was the ideal dose which increased chlorophyll, EPS extrusion and SMs degradation compared to non-treatment samples (0 g/g biomass). This dose was chosen for the second batch experiment, which evaluated the contribution of EPS and CaO_2 to SMs remediation (Fig. 2). Results of SDZ and SMZ were quite similar but differed from the SMX. SMs were removed at the highest degree with using only CaO_2. For SDZ and SMZ, EPS could remove SMs in the same degrees with CaO_2 at day 10. We conclude that the contributions of EPS and CaO_2 were very similar. For SMX, CaO_2 contributed 17.7%, while EPS removed 31.4%. However, when combining EPS and CaO_2, the removal efficacies reduced by 10 to 20% for all SMs. Possibly, this is accounted by the underlying interactions among EPS and the residual of CaO_2 in the reactors. This issue is explained later on in this paper.

[Insert Fig. 2]

The contributions made by each factor in SMs remediation are shown in Table 1. As previously explained, the cometabolism and CaO_2 contributed similarly to SDZ and SMZ remediation from 16.8 – 17.6%. For SMX, the cometabolism contributed twice more than CaO_2. The contributions of both cometabolism and CaO_2 were less than 20%. In turn, bioassimilation played an important role in SMs remediation in that it removed 24% of SDZ and SMZ, and accounted for 38% of SMX remediation. This meant that SMX accumulated in the microalgae cell to a greater extent than SDZ and SMZ (see section 3.5). We noted that photodegradation also contributed to SMs remediation, which accounted for 5-8% of the overall removal efficacies (Xiong et al., 2020). This scenario meant using a control reactor without microalgae in the reactor. In this study, microalgae presented in the reactors could reduce light transmittance in some extent due to the self-shading effect (Vo et al., 2018). This effect diminished the SMs photodegradation, so only a minor contribution was made here. Xiong et al. (2020) found that biosorption and hydrolysis of SMs were also negligible (<1%).

The SMs remediation occurred in 2 steps: SMs were cometabolized and then they were bioaccumulated and digested in microalgae cells. This study agreed with Park et al. (2017) who found that ammonia oxidizing bacteria accumulated more SMs than through cometabolism. The cometabolism rate of SMs was 0.058 L/g VSS.d, which was 2-fold less than the bioassimilation rate of 0.102 L/g VSS.d (Park et al., 2017). In contrast, Xiong et al. (2020) discovered that *Chlorella pyrenoidosa* could cometabolize 30 to 80% of total input SMX. Bioassimilation contributed insignificantly in that SMX was not detected in microalgae cells. In this study, CaO_2 pretreatment increased the bioassimilation of SMs by up to as much as 40%, meaning that it could deal with the high inlet concentration of SMs (200 μg/L).

Insert Table 1

3.4 Extrusion of extracellular polymeric substances and chlorophyll production by CaO_2 pretreatment

To validate the impact of CaO_2 pretreatment on microalgae, we analysed concentrations of EPS and chlorophyll at day 10 (Fig. 3a). The results indicated that CaO_2

pretreatment exerted a positive effect on microalgae. The EPS level of the CaO_2 pretreatment microalgae was three times higher than the non-pretreatment scenario. The concentration of soluble carbohydrate increased the highest in that it rose from 20 mg/L to 70 mg/L. Soluble protein also slightly shifted from 12 mg/L to 14.5 mg/L. The pretreatment also boosted the biochemical function of microalgae by elevating the chlorophyll concentration 1.5-fold. In the previous study, microalgae produced EPS and chlorophyll of 15 mg/L and 1.5 mg/L, respectively, in SMs-free environment (Vo et al., 2020a). Due to the stress of SMs and CaO_2 pretreatment, the ROS system has been stimulated to extrude more EPS and antioxidant enzymes. Consequently, EPS and chlorophyll concentrations increased 4 to 20 times. EPS was produced to scavenge the xenobiotic compounds in an extracellular environment such as antibiotics, plasticizer and non-steroidal anti-inflammatory drugs (Gonzalez-Gil et al., 2019; Olicón-Hernández et al., 2019; Wang et al., 2020a; Xiao and Zheng, 2016). Chlorophyll was also generated in the intracellular environment to alleviate the stress caused by hydroxyl radical groups. For example, due to the stress of carbamazepine, the *Spirulina platensis* extruded chlorophyll increasingly from 25 to over 30 mg/g biomass (Wang et al., 2020b).

To further understand the SMs cometabolism, we scanned the culturing liquid of the pretreatment and non-pretreatment samples by UV scanning, in the 240 to 400 nm range (Fig. 3b). The electron transfer (ET) band (240–400 nm) revealed the presence of benzene rings, polycyclic aromatic compounds (Korshin et al., 1997). The shapes of the two spectra were identical and showed high absorbance in the ET band region. This meant both samples contained high levels of aromatic rings, potentially from the SMs compounds. However, their absorbances occurred at different degrees. The absorbance of the pretreatment sample was less, which meant there was less benzene and aromatic ring than the non-pretreatment sample. The reason closely linked to fewer SMs compounds in the culturing liquid of the pretreatment sample. The benzene rings of those SMs compounds were cometabolized to ring-opening degradation products (Korshin et al., 1997).

[Insert Fig. 3]

3.5 Long-term assessment of sulfonamide remediation

We conducted the experiment in a continuous scenario (60 d) to assess SMs remediation. The reactors were operated in semi-continuous mode, in which the samplings and refills of nutrient and SMs were scheduled every 5 d (Fig. 4). Overall, SMs were removed in increasing amounts at a later stage. The results of our continuous experiment were consistent with the batch one. For example, the removal efficiencies of SDZ and SMZ increased gradually from 30% on day 5 to 60 – 80% by day 60. The removal efficiencies of three SMs compounds also differed from each other. SDZ and SMZ were removed at the highest level, i.e. 70-80%, while SMX was remediated at slightly less, at 20 – 40%. The impact of CaO_2 pretreatment only started to exert its effect on day 30. The pretreatment microalgae removed SMs 10 – 20% more than the non-pretreatment microalgae. Apart from increasing cometabolism activity, SMs did possibly accumulate in microalgae cells increasingly thanks to CaO_2 pretreatment. The evidence for this is reported in sections 3.6 and 3.7. The SMs removal efficacies of *Chlorella* sp. in this study were similar to the strain *C. vulgaris* of Kiki et al. (2020). With the initial concentration from 20 to 100 μg/L, the *C. vulgaris* remediated 75-85% of SMs (Kiki et al., 2020). The ammonia-oxidizing bacteria also removed SDZ from 80-90% via the cometabolism process (Wang et al., 2020a). The applied input concentrations of SDZ ranged from 10 to 100 μg/L, which were 2-fold less than this study.

[Insert Fig. 4]

3.6 Long-term assessment of EPS and enzymes production by CaO_2 pretreatment

The EPS and peroxidase enzyme extrusions were also assessed for long-term implications (Fig. 5). We measured EPS and peroxidase enzyme concentrations, which were generated in the culturing liquid and microalgae cells. In a long-term operation, protein

fractions in EPS were produced up to 90 mg/L, which were much higher than in the batch condition (15 mg/L). The pretreatment samples showed a higher protein level than the non-treatment samples. Protein level also shifted on day 35 compared to day 15. It is possible that the increase in protein fractions was due to adapting to the stress caused by SMs compounds. This outcome was closely linked to the higher removal efficacies of SMs after day 30. For the peroxidase enzyme, microalgae extruded more peroxidase enzyme in the culturing liquid than in the intracellular environment, around two times more. Compared with our previous finding, microalgae generated 1500-fold more peroxidase than the non-SMs samples, and 60–180 times more than the samples containing bisphenol A, tetracycline, and sulfamethoxazole (Vo et al., 2020b). We concluded that EPS and peroxidase enzyme extrusion was micropollutant-specific. Although peroxidase participated in SMs degradation, it was produced independently with CaO_2 pretreatment. The discussion of which enzymes involved in the SMs degradation can be found in section 3.7.3.

[Insert Fig. 5]

3.7 Mechanism of CaO_2 pretreatment advance SMs remediation

3.7.1 Degradation products of SMs using CaO_2 pretreatment microalgae

From section 3.5, it is clear that the SMs removal efficacies of the pretreatment samples were higher than the non-treatment in a continuous operation scenario. Cometabolism contributed around 17% to the remediation process. However, the impact of CaO_2 pretreatment in the long-term operation remained unclear. We noted that the contribution of bioassimilation was much higher than that of cometabolism. We screened out the degradation products and intensities of SMs and the degradation products in microalgae cells, which are summarized in Table 2.

Based on the input database of the suspected screening, we detected the degradation products of SMX mostly. A possible reason was that those degradation products of SMZ and SDZ were not included in the suspected list. Also, concentrations of these degradation products were less than the detection limit because SDZ and SMZ were remediated more efficiently than SMX, as shown in Fig. 4. The transformation products (TPs) of SMX were found using mass-to-charge ratios (m/z). The chemical structures were defined subsequently by identifying the fragment ions. We detected six metabolites of SMs, which agreed with other studies (Dudley et al., 2018, Kiki et al., 2020; Xiong et al., 2020). Of those degradation products, TP 213 possessed the highest peak intensity, suggesting that it was the major product of the SMs dissipation process. Results also demonstrated that the intensity of three SMs compounds and their metabolites of the pretreatment samples significantly exceeded the non-treatment samples. For example, we did not detect SDZ on day 15 and SMX on day 35. Particularly, the intensities of SMX of the pretreatment samples were 160 times lower than the non-treatment one. It meant the CaO_2 pretreatment enhanced the biochemical function of the intracellular environment of microalgae. In prior studies, metabolites of SMs were not detected in microalgae cells and plants (Chen et al., 2017; Xiong et al., 2020). We have found several degradation products of SMs in microalgae cells, and these were possibly attributed to strain-specific microalgae. As well, we demonstrated that CaO_2 pretreatment boosted the bioassimilation and digestion of SMs in microalgae cells.

The toxicity of SMs' degradation products is a particular challenge which needs to be addressed. Zhu et al. (2021) has demonstrated that the degradation products possessed higher lethal concentration than the parents' compounds which meant less toxic. Some of the degradation products generated from the cleavage of C-S/S-N and amino acid oxidation showed higher toxicity; however, those compounds presented at extremely low concentration like the TP 157 in our study. The impact to environment was therefore minimized.

[Insert Table 2]

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3.7.2 Propose the degradation pathways of SMs by CaO_2 pretreatment microalgae

Based on the above-detected degradation products, we proposed the degradation pathways of the SMs compounds as shown in Fig. 6. The degradation of SMX could occur in two stages (Xiong et al., 2020). At first, the oxidation and hydroxylation of amine group occurred and polarity and hydrophilicity of micropollutants were enhanced enough to increase SMs degradation. For the second stage, micropollutants were conjugated with polar compounds such as amino acid to shift the hydrophilicity of SMs to a higher level (Dudley et al., 2018). Specifically for the first step, the hydroxyl radical groups which were generated from enzyme activity possibly encountered on several bonds of the SMs compound. We hypothesized that there were five positions that enzymes, including peroxidase, could encounter on (marked 1 to 5 in Fig. 6). The 1, 4 and 5 positions were broken and then formed the hydroxylation products (TP 254). TP 254 was a degradation product originating only from SMX. The 2 and 5 positions related to ring-opening products such as TP 213. This was the main SMs degradation pathway because peak intensity of TP 213 was the highest. The cleavage of the aromatic ring in this degradation pathway reduced the toxicity load for microalgae (Kiki et al., 2020). For position 3, the amino group was cleaved and created TP 157. Unlike TP 254, TP 157 and TP 213 were the possible degradation products of SMX, SMZ, or SDZ. The detected degradation products and transformation pathways agreed with what other analyses reported (Huynh and Reinhold, 2019; Xiong et al., 2020). S atoms in the SMs compounds were the most sensitive compared to other atoms. According to the density functional theory calculation, the Fukui function of S atom was 6–45 times higher than the values of C atoms. This means that the reactivity of S atoms to enzyme degradation was also very high (Yin et al., 2017). The S-C and S-N were cleaved and formed the degradation products. However, the degradation products in this study resulted from the cleavage of the S-N bond only. This happened because the N atom was also in a weak position, which could be cleaved to generate nitroso- or nitro-groups (Yin et al., 2018).

[Insert Fig. 6]

In the second SMs remediation stage, SMX formed a pterin-related conjugation such as TP 429 (PtO-SMX). This product is a metabolite of pterin-SMX of which the primary amine group was converted to a keto one. Several studies have reported the formation of TP 429 during the SMX remediation process, which meant cometabolization by *Chlorella pyrenoidosa* strain, plant and bacteria (Huynh and Reinhold, 2019; Richter et al., 2013; Xiong et al., 2020). At first, SMX played two important roles in the biochemical system of microalgae: (i) inhibitor of the dihydropteroate synthase; and (ii) alternative enzyme substrate for the conjugation of pterin-sulfonamide (Richter et al., 2013). Initial pterin-sulfonamide products such as dihydropterin-SMX (TP 430) and Pterin-SMX (TP 428) were produced and then transformed into the terminal product TP 429. We did not detect any residuals of TP 430 and TP 428, which suggested that they were totally transformed into TP 429. Also, the glycosyl-SMX constituted a major degradation product, which accounted for up to 80% of the extractable metabolites, but they were not detected in this study (Huynh and Reinhold, 2019).
The Eawag-Biocatalyst/Biodegradation Database Pathway Prediction System (Eawag-BBD) was used to validate and compare the degradation pathways of SMs with other aerobic microbes (Fig. S7). This degradation pathway involved some enzymes such as cyclamate sulfamatase and sulfanilamide hydrolase (rule bt0144) and hydroxylase, monooxygenase and dehydrogenase (rule bt0036). The enzymes in rule bt0144 functioned the breakdown of sulfamate group to amine, and sulfanomide to amine and sulfonate. Also, the ones in rule bt0036 converted the aromatic methyl group to the primary alcohol (Eawag, 2019). Our degradation pathways agreed to Eawag-BBD on the cleavage of the aromatic rings to form the similar simpler products (e.g., TP 157 and product 3). However, we did not detect product 2 of Eawag-BBD (aromatic methyl) in our samples although we input this product in the analysis database. It indicated that microalgae preferably degraded the SMs to TP 213, rather than TP 157. It confirmed by the substantially higher peak area of TP 213 than the TP 157.

Another discrepancy linked to the conjugation products which were not included in the Eawag-BBD. It highlighted the distinct characteristics of microalgae compared to other aerobic microorganisms.

3.7.3 Which oxidative enzymes participate in SMs remediation?

The CaO_2 pretreatment increased SMs degradation via cometabolism and intracellular bioassimilation of microalgae. We observed that SMs degradation of the pretreatment sample was more proactive. Possibly, several enzymes were generated to digest SMs and the degradation products to a greater extent than the non-treatment samples (Vo et al., 2020b). Those enzymes, together with peroxidase, have cometabolized and metabolized SMs and reduced SMs-stress for microalgae. The involved enzymes for SMs detoxification could be micropollutants- or strain-specific (Ugya et al., 2020). For instance, we have demonstrated that the peroxidase enzyme was the one catalyzing SMs degradation. Other possible enzymes were the mixed-functional oxidases, including cytochrome P-450, cytochrome *b5*, and cytochrome P450 reductase (Wang et al., 2018b; Xiong et al., 2017). Those enzymes could either metabolize micropollutants through an integrative process or catalyze the conjugation of the large and polar compounds and micropollutants (e.g., glucuronic acid and glycine). Subsequently, degradation products were excreted to alleviate stress on microalgae (Huynh and Reinhold, 2019; Torres et al., 2008). Ferredoxin hydrogenase (EC 1.12.7.2), which belong to the membrane protein, could also participate in SMs degradation. This enzyme can reduce the N-O bond in the isoxazole ring and form ring-cleavage products such as TP 213 and TP 157 (Gonzalez-Gil et al., 2019; Mohatt et al., 2011).

3.8 Overall implications of using CaO_2 pretreatment for microalgae to remediate SMs.

The application of CaO_2 pretreatment for ant biotic remediation by microalgae has not been reported previously. This study explained the underlying mechanism of how CaO_2 pretreatment increased SMs remediation with evidence on the biochemical function and transformation pathways of SMs. This was the first study that demonstrated the potential of CaO_2 pretreatment of microalgae for full-scale application although further pilot- and full-scale systems-based researches are highly recommended. Optimizing the operating conditions is needed, such as wastewater matrix, types of antibiotics used, and microalgae strains. The hydraulic retention time of the continuous operation should be considered and shortened in the future works. CaO_2 pretreatment has been studied for nutrient and energy recovery in the wasted activated sludge. The offset cost from the received biogas amounted to as much as \$1.9 million in a full-scale wastewater treatment plant with a capacity of 10^5 m^3/d (Wang et al., 2018a). As shown in this study, antibiotics were remediated, and pigments such as chlorophyll were increasingly produced. This has much potential for the application of CaO_2 pretreatment using microalgae.

4. Conclusion

In this study, CaO_2 pretreatment of microalgae performed an effective SMs remediation in artificial wastewater. Pretreatment has conditioned microalgae to boost the functioning of the biochemical system. The ideal dose for treatment was 0.1 g CaO_2/g microalgae. The pretreatment microalgae remediated SMs much better than the non-pretreatment, by more than 10 to 20% since day 30. We concluded that microalgae have adopted to the CaO_2 pretreatment and started remediating SMs more efficiently. Interestingly, pretreatment facilitated an extensive accumulation and digestion of SMs in the intracellular environment, up to 160-fold higher than the non-pretreatment scenario. Peroxidase is a particular enzyme participating in the cometabolism and degradation of SMs to several metabolites including ring cleavage, hydroxylation and pterin-related conjugation.

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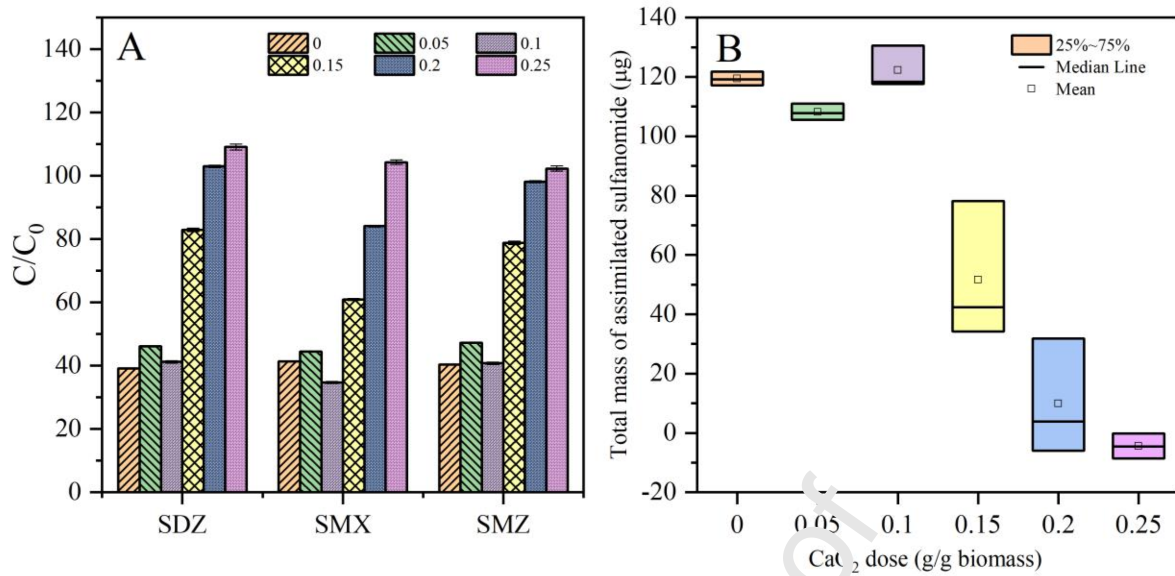

Fig.1. Remaining SMX, SMZ, and SDZ in the effluent (Panel A) and total mass of SMX, SMZ, and SDZ removed (Panel B) in each CaO_2 dose at day 10. C represents the concentrations of SMs at time t, C_0 represents the concentrations of SMs at the starting time. Box plots in panel B contain the mass of SMX, SMZ, SDZ which were removed. The reactors were dosed with SMZ, SMX, and SDZ (each 200 µg/L). All measures showed significant differences ($p < 0.05$) amongst CaO_2 doses (n=2).

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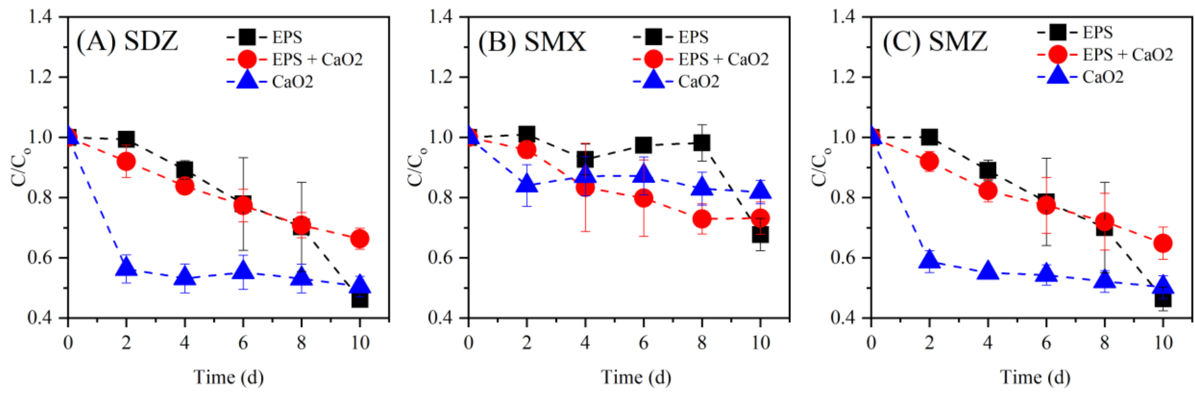

Fig. 2. Remaining SDZ (Panel A), SMX (Panel B), and SMZ (Panel C) in the effluent of dose 0.1 g/g biomass. The y axis of all panels performs the ratio of tested concentration (C) and initial concentration (C_o). The x axis represents time course from day 0 to day 10. The reactors were dosed with SMZ, SMX and SDZ (each 200 μg/L). All measures showed significant differences ($p < 0.05$) (n=2). The reactors did not contain microalgae biomass but only extracted EPS and CaO_2.

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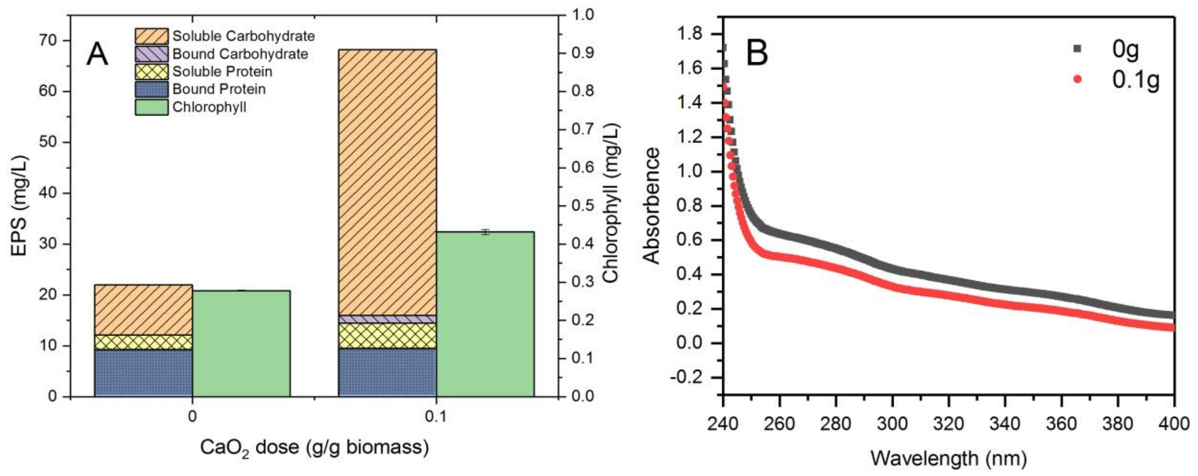

Fig. 3. EPS and chlorophyll production by microalgae (Panel A) and UV scan of supernatant (Panel B) using 0 g and 0.1g CaO_2/g biomass doses. Panel A includes double y axis: EPS concentration (left) and chlorophyll concentration (right). All measures showed significant differences ($p < 0.05$) amongst CaO_2 doses (n=2).

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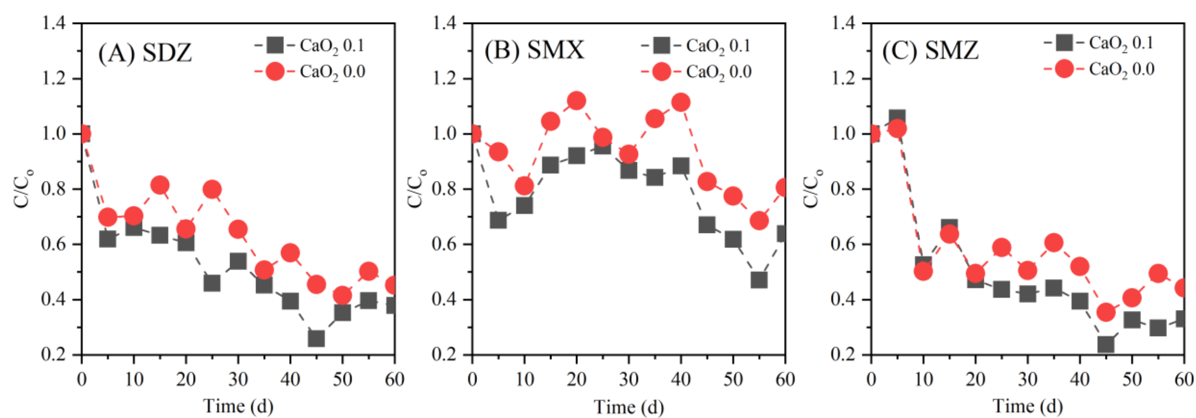

Fig. 4. SDZ (Panel A), SMX (Panel B), and SMZ (Panel C) removal by microalgae in a continuous scenario, dosing 0 g and 0.1 g CaO_2. The y axis of all panels represents the ratio of tested concentration (C) and initial concentration (C_o). The x axis represents the passing of time from day 0 to day 60. All measures showed significant differences ($p < 0.05$) amongst CaO_2 doses (n=2).

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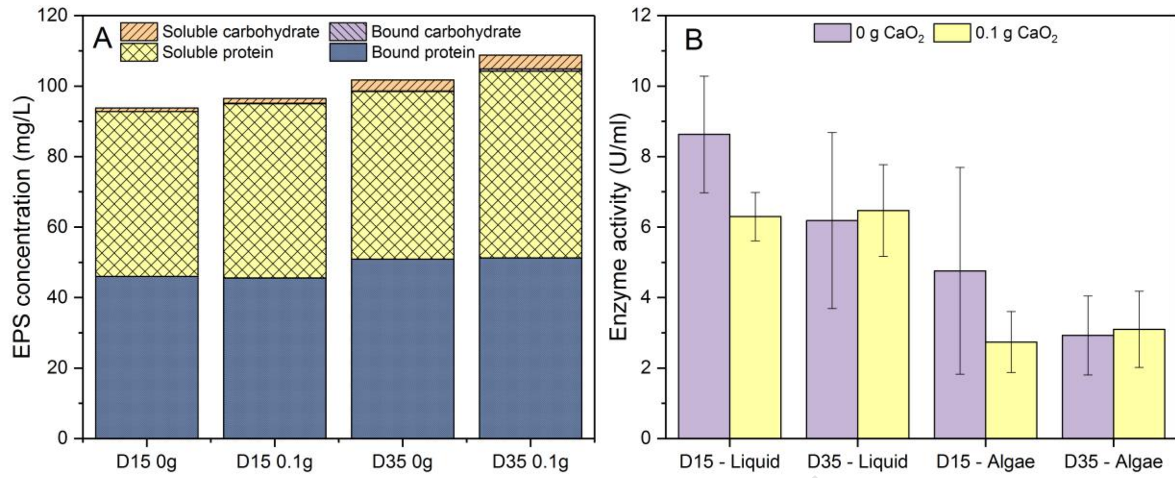

Fig. 5. EPS (Panel A) and peroxidase (Panel B) generation by microalgae using 0 g and 0.1 g CaO_2/g biomass doses at days 15 and 35, respectively. Peroxidase activity was measured in the culturing liquid and intracellular microalgae cells. All measures in panel A revealed significant differences ($p < 0.05$) amongst CaO_2 doses. Measures in panel B amounted to only insignificant difference ($p>0.05$). Data were measured in duplicate (n=2).

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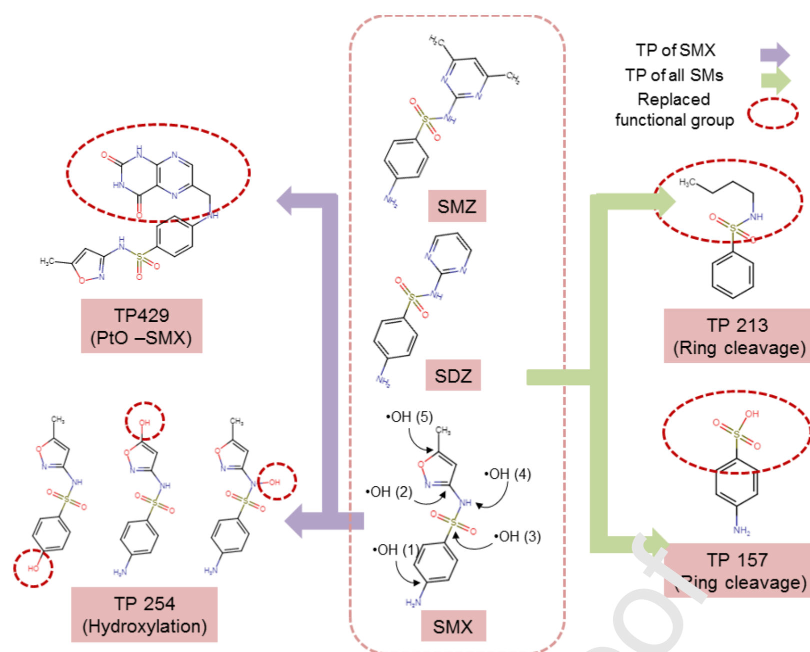

Fig. 6. The proposed structures of TPs and degradation pathways of SMs to 6 degradation products (TP 254, TP 213, TP 430 and TP 157). Five positions which peroxidase enzyme encountered on the SMs molecules are numbered 1 to 5 in the parentheses.

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List of tables

Table 1. Contribution of different factors to SMs remediation using a 0.1 g CaO_2/g biomass dose

Factors (%)	SDZ	SMX	SMZ
C_C (Cometabolism)	16.8	17.4	17.6
C_{CaO2}	16.8	9.8	17.6
C_b (Bioaccumulation)	24.3	37.9	23.7
C_r (Remaining in the aqueous phase and adsorbed on cells surface)	42.1	34.9	41.1
Total	100	100	100

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Table 2. Peak intensities of SMs compounds and the degradation products in cells of microalgae for bioaccumulation, collected at days 15 and 35 of the continuous experiment.

Detected compounds	Potential parent compounds	D15-0g	D15-0.1g	D35-0g	D35-0.1g
SDZ	-	11567	n.d	n.d	n.d
SMZ	-	n.d.	n.d	n.d	n.d
SMX	-	433004	2763	8987	n.d
TP 254 -1	SMX	20233	7711	21140	17920
TP 254 -2	SMX	20218	10528	17614	15110
TP 254 -3	SMX	84165	n.d.	17844	43240
TP 429	SMX	45591	52004	51387	n.d.
TP 157	SMX, SMZ, SDZ	3315	5117	4355	n.d
TP 213	SMX, SMZ, SDZ	109398	77158	90327	99334

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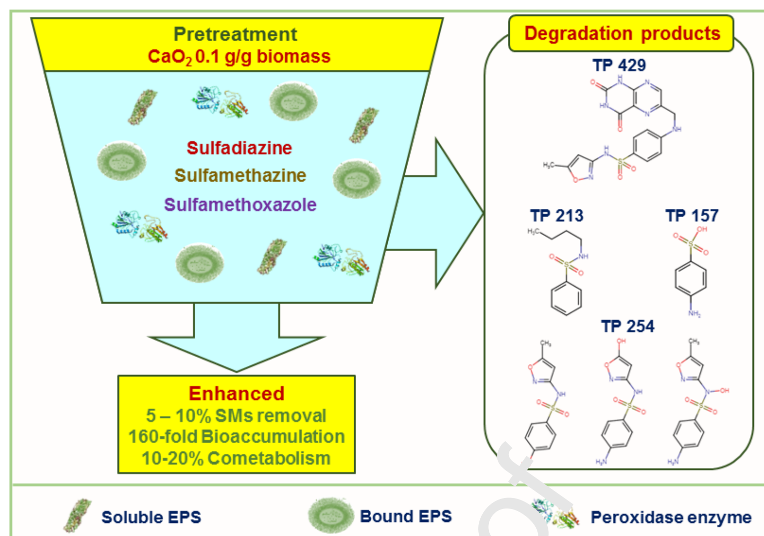

Graphical abstract

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Highlights

- Dose of 0.05-0.1 g CaO_2/g biomass removed 60% of the spiked sulfonamide antibiotics.
- CaO_2 pretreatment increased cometabolism of sulfonamides 10 to 20%.
- Bioassimilation removed 24% sulfadiazine and sulfamethazine, and 38% sulfamethoxazole.
- Ring cleavage, hydroxylation and pterin-related conjugation metabolites were detected.
- Peroxidase is a micropollutants-specific enzyme for sulfonamides cometabolism.

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