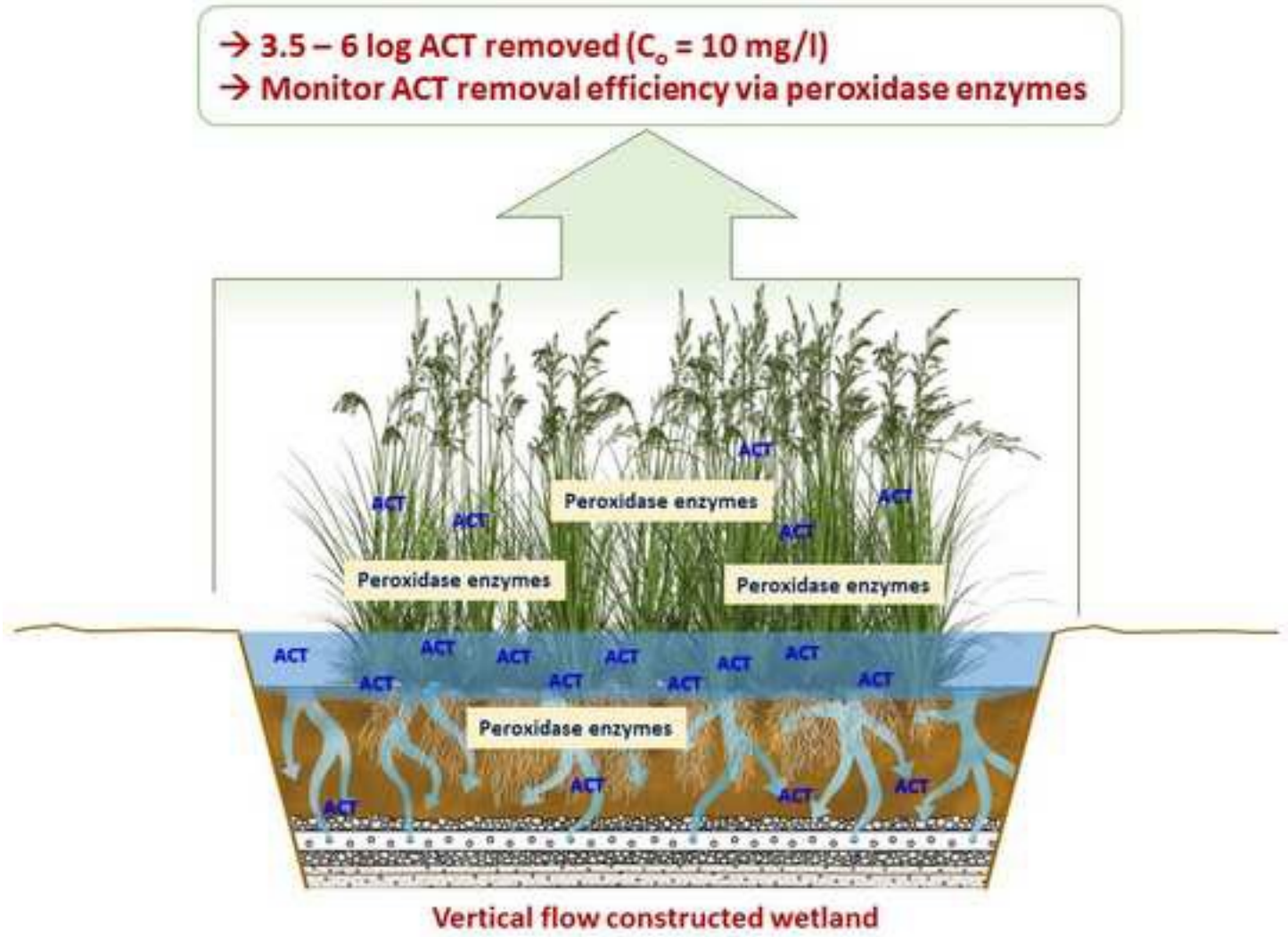
Graphical Abstracts



Highlights

CW removed 3.5 to 6 log ACT of initial concentration 10 mg/l.

ACT concentration in CW effluent was safe for drinking water.

Peroxidase enzymes could monitor ACT removal efficiency via first order equation.

Removal and monitoring acetaminophen-contaminated hospital wastewater by vertical flow constructed wetland and peroxidase enzymes

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Abstract

Hospital wastewater contains acetaminophen (ACT) and nutrient, which need adequate removal and monitoring to prevent impact to environment and community. This study developed a pilot scale vertical flow constructed wetland (CW) to (1) remove high-dose ACT and pollutants in hospital wastewater and (2) identify the correlation of peroxidase enzyme extruded by *Scirpus validus* and pollutants removal efficiency. By that correlation, a low-cost method to monitor pollutants removal was drawn. Plants, such as *Scirpus validus*, generated peroxidase enzymes to alleviate pollutants' stress. Results showed that the CW removed 3.5 to 6 logs of initial concentration 10 mg ACT/L to a recommended level for drinking water. The CW eliminated COD, TKN and TP efficiently, meeting the wastewater discharged standards of Thailand and Vietnam. By various multivariable regression models, concentrations of ACT in CW effluent and enzymes in *S. validus* exhibited a significant correlation (p<0.01, R^2=68.3%). These findings suggested that (i) vertical flow CW could remove high-dose ACT and nutrient and (ii) peroxidase enzymes generated in *S. validus,* such as soluble and covalent ones, could track ACT removal efficiency. This would help to reduce facilities and analytical cost of micro-pollutants.

Keywords: Acetaminophen, Constructed wetland, Hospital wastewater, Nutrient, Peroxidase enzyme, Micro-pollutant.

1. Introduction

Hospital wastewater contains various pollutants such as micro-pollutants and nutrient. Micro-pollutants can accumulate in the human body via contaminated drinking water and food; then posing a health risk to community. Among micro-pollutants, acetaminophen (ACT) emerges regularly in hospital wastewater because it is one of the most prescribed drugs recently (Phong Vo et al., 2019). In France and Spain, ACT was consumed at highest rates of 54.3 and 22.6 g/y. inhabitant, respectively (Ortiz de García et al., 2013). In 2004, ACT was the most prescribed medication in Taiwan of 600 million doses (Lin and Tsai, 2009). It is detected frequently in hospital wastewater treatment plants across Asia, Europe and America with notable concentrations (50 - 400 µg/L) and frequency (100%) (Kosma et al., 2010; Kumar et al., 2019). This concentration is much higher than the recommended level for drinking water (71 ng/L) (Vulliet and Cren-Olivé, 2011). Nutrient is another pollutant of concern in hospital wastewater. High concentration of nutrient can cause eutrophication in water reservoir. Critically, hospital wastewater needs adequate treatment to remove ACT and nutrient before discharging to water reservoir.

Constructed wetland (CW) can resolve the pollutants induced by hospital wastewater. This technology functions by infusion of biological, physical and chemical processes. Those processes co-occur in CW and enhance pollutants removal extensively (Hickey et al., 2018; Zhang et al., 2014a). CW also certifies a low-cost technology for decentralized wastewater treatment system. Its operation and maintenance cost 0.014 – 0.0134 \$USD/m^3 wastewater compared with 0.1151 – 0.2465 \$USD/m^3 wastewater of conventional system (Arias and Brown, 2009; Chen et al., 2008). Practically, CW includes vertical and horizontal flow configuration. The vertical flow CW is more competent for hospital wastewater treatment because it possesses advanced properties. For example, the vertical flow CW conditions nitrifying ammonia and oxidation process effectively (Vymazal, 2011). In terms of footprint,

58 the vertical flow CW uses 1-3 m^2/population equivalent, whereas the horizontal flow CW
59 requires 5 m^2/ population equivalent (Vymazal, 2011).

60 In CW, a plant can uptake and remove pollutants. Pollutants accumulate in the plant's body,
61 causing stress and altering the plant's biochemical system. This induces plant to generate
62 reactive oxygen species (e.g. H_2O_2) to signal the endangered situation (Zandalinas and
63 Mittler, 2018). However, the overproduction of reactive oxygen species can damage the
64 macromolecules such as nucleic acids, proteins and lipids. To alleviate the situation, the plant
65 triggers the antioxidant system (Jaskulak et al., 2018). The antioxidant system includes
66 peroxidase enzymes of soluble (SP), ionical (IP) and covalent (CP) forms that localized as
67 soluble, ionical and covalent bound to cell wall. SP enzyme presents in apoplastic fluid and
68 penetrates through cell walls. IP enzyme exists in hydrophobic and ionic conditions with
69 polysaccharides and proteins while CP enzyme cross-links with the cell wall components by
70 covalent bonds. Peroxidase enzymes are catalysts for H_2O_2 to oxidize organic compounds
71 and therefore reduce stress to plant. The peroxidase undergoes a cyclic reaction as it reacted
72 the phenolic compound (Eq. 1-3). The peroxidase induces reactions in its original form, then
73 oxidized by H_2O_2 to form the intermediate ($Peroxidase_1$). The intermediate E1 oxidizes
74 phenolic substances (PhOH) to free racial (PhO) and next intermediate ($Peroxidase_2$). The
75 intermediate $Peroxidase_2$ continually oxidizes phenolic compounds and returns to the native
76 form, ending the cycle (Francoz et al., 2015).

77 $Peroxidase + H_2O_2 \rightarrow Peroxidase_1 + H_2O$ (Eq. 1)

78 $Peroxidase_1 + PhOH' \rightarrow Peroxidase_2 + PhO$ (Eq. 2)

79 $Peroxidase_2 + PhOH'' \rightarrow Peroxidase + PhO + H_2O$ (Eq. 3)

80 Although CW is used widely for wastewater treatment, research gaps remain in the
81 application of vertical flow CWs. First, the actual ACT-removal capacity of vertical flow CW

82 is underestimated in previous studies experimented with a low-range ACT concentration (i.e.
83 less than mg/L) (Ávila et al., 2014; Petrie et al., 2018; Yi et al., 2017). Still, the quantitative
84 correlation of peroxidase enzymes and pollutants in wastewater is not considered. Several
85 studies report that peroxidase involves in the phenolic compounds degradation process (i.e.,
86 ACT, diclofenac, bisphenol A). For instance, peroxidase and glycosyltransferase enzymes are
87 proved as catalysts for clofibric acid degradation, but their correlation of peroxidase enzyme
88 and the pollutants removal is not adequately quantified (Dordio et al., 2009; Huber et al.,
89 2012; Huber et al., 2016). Herein, to explore the correlation, we hypothesize that pollutants of
90 hospital wastewater, including ACT, correlate with H_2O_2, SP, IP and CP enzymes as first-
91 order linear model. By establishing that correlation, peroxidase enzymes can track pollutants
92 removal efficiencies of CW. Hence, analytical cost of pollutants is saved extensively.

93 To unveil those gaps, the objectives of this work are to (1) investigate high-dose ACT
94 removal (10 mg/L) and (2) monitor pollutants removal efficiencies by peroxidase enzymes in
95 a pilot scale vertical flow CW.

96 ## 2. Materials and methods

97 ### 2.1 A vertical flow CW and its operation

98 The pilot scale vertical flow CW was constructed using respective length, width and height of
99 1.5, 0.6 and 0.6 m. The media bed contained sand, pea gravel and gravel with respective
100 height of 0.1, 0.2 and 0.4 m from top to bottom. The porosities of sand, pea gravel and gravel
101 bed were different in d_{10} and d_{60} values (Table S1). These differences made the filtration bed
102 with pore size from small to large from top to bottom. The bottom of CW was sloped 1% for
103 drainage. The *Scirpus validus*, which grew naturally in local wetlands, was selected for this
104 CW. It was planted in the CW for three weeks to adapt and grow in new environment.

105 The CW was operated continuously for 65 d using flow rate of 75 - 85 l/d, coupling hydraulic
106 retention time of 5 d. The CW was fed in which the water surface was 0.05 m below the sand
107 surface. Wastewater for this experiment was influent of a hospital's wastewater treatment
108 plant (Pathumthani, Thailand). The concentrations of suspended solid (SS), chemical oxygen
109 demand (COD), NH_4^+-N, NO_3^--N, total Kjeldahl nitrogen (TKN) and total phosphorus (TP)
110 in wastewater were 500 ± 236.8, 352.7 ± 164.1, 25 ± 6.4, 1.0 ± 0.6, 36.6 ± 12.6, 7.9 ± 4.3
111 mg/L, respectively (n=4). Wastewater was stored in 1 m^3 tank and mixed continuously during
112 the feeding.

113 ACT concentration in wastewater was stable at 2.7 ± 0.83 μg/L (n = 4). To evaluate ACT
114 removal efficiency by the CW, ACT concentration was increased to 10 mg/L by adding a
115 stock solution (Sigma Aldrich, Thailand).

116 2.2 Methods

117 2.2.1 Plant and wastewater sampling

118 The *S. validus* plant and wastewater samples were collected every 5 d. For plant samples,
119 root biomass was conserved by removing sand around the plant and gently pulling from CW.
120 All collected plants were rinsed under deionized water for 2 min, air dry in room temperature
121 and stored at 4 ^oC prior to analysis. The wastewater samples were collected via the bottom
122 valve of CW. The samples were preserved using Ethylenediaminetetraacetic acid (EDTA) to
123 prevent microbial activity until analysis.

124 2.2.2 Acetaminophen analysis

125 ACT analysis was described in our previous work (Phong et al., 2016). In brief, solid-phase
126 extraction was performed on Oasis HLB sorbent cartridges. The cartridges were
127 preconditioned with 4 ml of methanol and 6 ml of distilled water (pH =3.5). The water
128 samples were percolated through the cartridges at flow rate of 5 ml/min. To analyse ACT, the

cartridges were eluted with 6 ml of methanol into 10 ml test tube. Methanol was evaporated under a gentle nitrogen stream at 37 ºC and reconstituted with acidified ultra-pure water (0.01% formic acid: = 9:1) to final volume of 1 ml. Final extracts were stored in 2 ml glass vials and analyzed by HPLC-MS/MS (Shimadzu, 8060).

2.2.3 Hydrogen peroxide analysis

Hydrogen peroxide (H_2O_2) was analysed as described by Phong et al. (2016). Plant samples were homogenized by using 1.5 g (wet weight), ground in mortar with liquid nitrogen. Then, they were suspended in 5 ml of 0.2 M perchloric acid and centrifuged at 1200 g, 4 ºC for 5 min. The received supernatants were neutralized by 4 M KOH to pH 7.5. The total volume of each sample was 10 ml. The sample was centrifuged at 3000 g, 4 ºC in 15 min to remove insoluble potassium perchlorate. Subsequently, 800 µl of aliquot was applied to 0.12 g anion exchange resin column (AG 1-X2, Bio-Rad). The column was washed by 3.2 ml distilled water before collected 1 ml of elute.

In spectrophotometer cuvette, the elute was added 400 µl of 12.5 mM 3-dimethylaminobenzoic acid, 80 µl of 1.3 mM 3-methyl-2-benzothiazolinone hydrazone and 20 µl horseradish peroxidase, respectively. The reaction mixture was incubated at 25 ºC for 5 min. Then the reaction was stopped by cooling in ice bath for 15 min. After 10 min, the absorbance was read at 590 nm and compared with calibration curve for result (Table S2).

2.2.4 Peroxidase analysis

Peroxidase fractions were extracted by the following steps. Initially, 0.5 g of plant tissue was ground using 4 ml of 50 mM Tris Maleate (pH 6.0). The solution was transferred to centrifuge tube, kept immediately in triturated ice and centrifuged at 2 ºC, 1000 g in 10 min. The supernatant was collected stored in freezer at -80 ºC. This fraction was for measuring the SP enzyme. The precipitate was kept for extraction of IP and CP enzymes.

Of the SP enzyme, 50 µl of plant extract (supernatant from the above step) and the chemicals were added in cuvette consisting of 500 µl of 30 mM hydrogen peroxide, 500 µl of 168 mM guaiacol, 1.95 ml of 40 mM tris maleate buffer pH 6.0, respectively. The cuvette was read at absorbance 470 nm.

$$\text{SP enzyme activity} = Abs_{60} - Abs_0 \quad \text{(Eq. 4)}$$

Where

Abs_{60}: absorbance at time 60 s

Abs_0: absorbance at time 0

The IP enzyme was extracted from the precipitate of SP enzyme. The precipitate was incubated by 2 mL of 0.2 M $CaCl_2$ in room temperature for 2 h. The mixture was centrifuged at 2 ^oC, 1800 g in 20 min.

The CP enzyme was extracted from the precipitate of IP's extraction process. The precipitate was added 1 ml of triz maleate buffer 40 mM. Then, it was centrifuged at 2^oC, 1800 g in 20 min. The supernatant was collected for CP. The measurement of CP and IP enzyme activity was similar to the procedure of SP's analysis. One unit of peroxidase is defined as the amount of enzyme that reduces1.0 mmole of H_2O_2 per minute at 37 °C.

2.2.5 Analysis of other parameters

The other parameters including suspended solid (SS), chemical oxygen demand (COD), total kjeldahl nitrogen (TKN), ammonia nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), total phosphorus (TP) were analyzed according to the Standard Method (APHA., 2005).

2.2.6 First-order kinetic modelling

By assuming that CW was a continuous stirred-tank reactor, the first-order kinetic model was used for ACT removal (Eq. 5). The volumetric decay rate constant (k_v) was estimated based on inlet–outlet data of the CW. The first-order reaction equation was:

$$\frac{C_{out}}{C_{in}} = k_v \times t \quad \text{(Eq. 5)}$$

Where

C_{out}: pollutant concentration in the effluent (μg/L)

C_{in}: pollutant concentration in the influent (μg/L)

k_v: volumetric decay rate constant (/d)

t: time (d)

2.2.7 Multi-variable regression

The multi-variable regression analysis was employed for establishing the correlation of enzymes and pollutants concentrations in effluent of CW. Firstly, the Lindeman, Merenda and Gold analysis was conducted for evaluating the importance of variables and significance of models. Only the respective models and variables possessed significant R^2 and contribution values would be processed. Then, the multivariable regression methods, including “all possible subsets”, “forward selection” and “backward elimination”, were implemented to establish models.

The multivariable regression model is assumed as the following:

$$y = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \ldots + \beta_n x_n \quad \text{(Eq. 6)}$$

Where

y: responsible variable ($C_{X.E}$)

x_i: predictor variables ($C_{X.R}$,$C_{X.S}$)

$C_{X.E}$: concentration of pollutants X, including ACT, COD, NO_3^--N and TP, in the effluent of CW.

$C_{X.R}$, $C_{X.S}$: concentration of enzymes X, including H_2O_2, SP, CP and IP in the root and shoot of plant, respectively.

The R software was applied for multivariable regression using the Relaimpo and Mass package. The Relaimpo package was for Lindeman, Merenda and Gold analysis, while Mass was to operate the "all possible subsets", "forward selection" and "backward elimination" methods. The code was provided in appendix.

2.3 Statistical analysis

The analyses of variance (ANOVA) were used for statistical analysis. The repeated measures ANOVA were applied to investigate the significant difference of enzymes in root and shoot. Concentrations and removal efficiencies were presented as mean ± standard deviation. All the statistical analyses were performed by R software. The statistical differences of results were compared by using means' values with 95% confidence level.

3. Results and discussions

3.1 Acetaminophen removal by constructed wetland

By employing 10 mg ACT/L concentration in the influent, we found that this vertical flow CW reduced 3.5 to 6 log ACT. The ACT concentration in CW effluent decreased to below 0.4 μg/L and stayed consistently from day 15^{th}-25^{th} (Fig. 1a). This concentration was safe to aquatic living given the standard EC_{50} concentration – a parameter for assessing toxicity - was 50 mg/L (Kim et al., 2007). It also complied with recommendation for drinking water (71 ng/L) (Vulliet and Cren-Olivé, 2011). After 45 d, ACT concentration in CW effluent was

less than the suggested level for drinking water. Therefore, this vertical flow CW compromised high-dose ACT and removed it effectively.

A removal kinetic and half-life ACT degradation were analysed to quantify the technical performance of CW. As a result, the ACT removal kinetic of CW fitted highly to first order (R^2=0.89) and half-life degradation was 13.6 d (= —— (Fig. 1b). The half-life

degradation values were higher than previous reports, which documented from 0.3 to 2.1 d (Ranieri et al., 2011; Yamamoto et al., 2009). The discrepancy was attributed to the applied wastewater sources and initial ACT concentrations. Those authors used distilled and river waters, which unanticipated the side effects of other factors in hospital wastewater (e.g. high suspended solid level). In addition, the half-life of ACT degradation depended largely on its initial concentration such as 10 mg/L in this study compared with 0.7 to 100 µg/L (Ranieri et al., 2011; Yamamoto et al., 2009).

[Insert Fig. 1]

This vertical flow CW could eliminate high-dose ACT as it was regulated concurrently by various mechanisms, encompassing plant uptake, biodegradation and adsorption (Phong et al., 2016). Plant uptake advanced pollutants removal in CW. *S. validus* could uptake 16.8 - 58.1 µg ACT/g fresh weight.d and degrade ACT to non-toxic metabolites (Phong et al., 2016). For example, ACT and its metabolites were detected in plant's tissue of *Armoracia rusticana* and *Brassica juncea* (Bartha et al., 2010; Huber et al., 2009). The uptaking process impaired 70% of 1mM ACT dose in 3h. After 6h, 18% paracetamol, 64% paracetamol–glucoside, 17% paracetamol glutathione and 1% of cysteine conjugate were detected in plant tissue (Huber et al., 2009). Biodegradation was a well-established removal process because aerobic and anaerobic bacteria in CW could assimilate pollutants. For instance, *Pseudomonas* spp. and *Bacillus* spp accumulated 1.0 to 4.1 mg ACT/$g_{biomass}$.h (Baratpour and

242 Moussavi, 2018). Microorganism could be inhibited by high-dose ACT - 50 to 1000 mg/L
243 (Alvarino et al., 2014); nevertheless those concentrations unlikely existed in wastewater.

244 For adsorption, it removed ACT ineffectively since ACT was a low hydrophobic substance
245 (K_d<3) (Zhang et al., 2014a). Adsorption also could not compete with biodegradation. It
246 accounted only 30% ACT removal in the co-processes of adsorption and biodegradation (Lin
247 et al., 2010). To improve ACT adsorption efficiency, we suggested using light expanded clay
248 aggregates media, so-called LECA, as this material contains alkaline of oxides and
249 carbonates (Machado et al., 2017). They would increase adsorption efficiency by enhancing
250 electrostactic interaction of LECA's surface and pollutants.

251 This vertical flow CW proceeded horizontal flow CW in ACT removal (Table 1). Typically,
252 horizontal flow CW removed beyond 99% ACT load; however ACT initial concentration was
253 considerably low at 750 ng/L and flow rate at 1 m^3/d that much less than this work (Ranieri et
254 al., 2011). In another study, it removed 45% ACT operating with 30 ng ACT/L in influent
255 and achieving 16 ng ACT/L in effluent (Verlicchi et al., 2013). Similarly, horizontal CW
256 used various substrates (e.g., steel slag, gravel) and removed only 65% ACT of initial
257 concentration 273 ng/L (Petrie et al., 2018). For the reason, horizontal flow CW just
258 exploited part of its media and plant bed because wastewater was fed on side, rather than the
259 whole surface like vertical flow CW. This vertical flow CW removed high-dose ACT better
260 also thanks to *S. validus*. This plant could uptake 80% micro-pollutants - clofibric acid - at
261 notable dose 2 mg/L (Zhang et al., 2013). Other plants, such as *Typha* spp., removed only
262 50% clofibric acid at lower dose 20 μg/L (Dordio et al., 2009). Although vertical flow CW
263 displayed a distinct ACT removal efficiency in this work, horizontal flow CW also needed
264 studies with high-dose ACT for a fair comparison.

265 [Insert Table 1]

266 3.2 Nutrient removal in constructed wetland

267 The vertical flow CW effectively removed nutrients (Table 2). It diminished sufficiently 80%
268 SS, NH_4^+-N and COD. The process also reduced at least 65% TKN and TP. Although the
269 system unlikely removed TN and TS as that much; nevertheless, TSS, NH_4^+-N, NO_3^--N,
270 TKN, TN, TP, COD concentrations in CW effluent complied with discharged standards for
271 hospital wastewater in developing countries: Vietnam (MonRe Vietnam, 2010) and Thailand
272 (MonRe Thailand, 2005) (Table S3).

273 In this study, vertical flow CW removed TN moderately, less than 22%, because of
274 insufficient denitrification (Sgroi et al., 2018). In essence, the horizontal flow CW
275 conditioned denitrification better than vertical flow pattern. For example, horizontal flow CW
276 removed above 50% TN of initial concentration 50-200 mg/L (Nguyen et al., 2018). Our
277 results agreed with previous findings. The vertical flow CW could handle 35-52% TN while
278 horizontal flow one removed 69% TN (Sgroi et al., 2018). Similarly, Kahl et al. (2017)
279 reported that vertical flow CW treat 45-56% TN compared with 52-72% TN of horizontal
280 flow CW (Kahl et al., 2017). To increase TN removal in this study, we proposed applying
281 consecutive vertical flow CWs or vertical-horizontal hybrid CWs. If spacing was limited,
282 recirculating 50% effluent would shift TN removal 66% (Ávila et al., 2017).

283 For solid pollutant, high TS concentration remained in the effluent because of dissolved solid
284 in hospital wastewater (Carraro et al., 2016). Those dissolved solids included salts, solvents
285 and hydrocarbons. In CW, biological process performed poorly at high-dose TDS and plant
286 uptake demonstrated a fair removal efficiency, which exclude 21% of 2500 mg TDS/L
287 (Valipour et al., 2014). Hence, we suggest using advanced oxidation process to diminish
288 high-dose TDS, but it would generate extra cost. Otherwise, LECA media was an alternative
289 to augment both TN and dissolved solids removal (Liu et al., 2014; Machado et al., 2017).

290 This media was highly useful for removing water-soluble compounds in hospital wastewater,
291 such as furosemide, benzene, alcohol and acid.

292 The rising concern was whether other pollutants influenced ACT removal efficiency in CW.
293 In practice, the bulk nutrients would nurture plant and microbial community growing up and
294 augment ACT removal efficiency accordingly (Zhang et al., 2014a). Rhizosphere and
295 microbial community played an important role in creating the aerobic environment and
296 uptaking ACT. The suspended solid concentration of wastewater also partly adsorbed ACT
297 (Zhang et al., 2014a). Hence, ACT removal received indirect benefits from other pollutants of
298 hospital wastewater.

299 [Insert Table 2]

300 3.3 Hydrogen peroxide enzyme response to pollutants stress

301 The plant produces H_2O_2 enzyme in apoplast, chloroplasts, mitochondria. The invasive
302 targets of H_2O_2 are nucleic acids, proteins and lipids (Zandalinas and Mittler, 2018).
303 Environmental stresses, such as excess light, UV, drought, high temperature and pollutants
304 trigger H_2O_2 production by various signalling pathways. In this study, we recorded H_2O_2
305 concentrations in shoot and root of *S. validus,* stressed by hospital wastewater containing
306 ACT and nutrient, to elucidate its response.

307 After exposure to hospital wastewater, the shoot of *S. validus* generated 636 ± 130.1 nmol
308 H_2O_2/g FW (n=14) while the root produced 426.8 ± 69.6 nmol H_2O_2/g FW (n=14) (Fig. 2a).
309 H_2O_2 concentration of the shoot was higher because the plant translocated ACT to the shoot
310 after uptaking it. The shoot would degrade ACT to metabolites, such as glucoside,
311 glutathione and cysteine conjugate (Huber et al., 2009). For small-dose micro-pollutants less
312 than 100 μg/L, the root could degrade them totally; thus, H_2O_2 concentration in the root
313 exceeded the shoot (Sun et al., 2018). One strategy to alleviate the stress was to translocate

pollutants from root to shoot but it depended on the originality of pollutants. For example, Pb is a heavy metal possessing high atomic number and its mobility is the lowest amongst all heavy metals. Thus, Pb is mostly accumulated in the root than the shoot (Gupta et al., 2013). For ACT, it can be seen it was an easy-translocating compound for plant. It explained for the competitive H_2O_2 level in shoot compared to root in both hydroponic and hospital wastewater (Phong et al., 2016).

In hydroponic conditions, shoot and root produced 318.5 ± 64.5 nmol/g FW and 442.2 ± 48.6 nmol/g FW, respectively (Phong et al., 2016). They were a half to equivalent compared with H_2O_2 concentration experimented with hospital wastewater. Hospital wastewater contained high-dose ACT and other pollutants (e.g., nitrogen, carbon, phosphorus) that would shift H_2O_2 concentration consequently. ACT is a toxic organic substance rather than a nutrient. Its EC_{50} dose to macrophytes was documented at 450 mg/L (Nunes et al., 2014). As known, plant produced H_2O_2 by various factors, not only ACT; however, comparing the studies of artificial (low-dose ACT) and hospital wastewater (high-dose ACT), we indicated that the stress of ACT to plant dominated other pollutants because, due to the presence of ACT, H_2O_2 concentration in artificial wastewater accounted from a half to similar level H_2O_2 in hospital wastewater.

3.4 Peroxidase enzyme response to pollutants stress

Plant can produce several enzyme types such as superoxide dismutase, catalase and peroxidase. Peroxidase is special enzyme of the plant as it involves directly to the degradation of phenolic compounds by catalysing or oxidizing them, supporting by H_2O_2. Peroxidase enzyme is so-called ROS-consuming and ROS-generating as co-function. Other enzymes did not participate in the phenolic compound degradation process. For example, the superoxide dismutase scavenges the dismutation of the superoxide radical to ordinary molecular oxygen or hydrogen peroxide, and the catalase decomposes H_2O_2 to water and

339 oxygen. By those functions, peroxidase interrelates micropollutants degradation process and
340 it includes ACT (Huber et al., 2012; Huber et al., 2016). Peroxidase is the most active and
341 dynamic one against micropollutants. Recent proteomic studies showed that peroxidase
342 accounts half of the oxido-reductase class in plant (Francoz et al., 2015). Oxido-reductase is
343 the main oxidative class functioning the organic compounds' degradation (Francoz et al.,
344 2015). Therefore, peroxidase participates in ACT degradation process competitively than
345 other enzymes. Under normal condition, peroxidase enzymes engage in lignin and phenolic
346 polymers synthesis. Upon suffering from H_2O_2 stress, peroxidase enzymes would catalyse the
347 degradation of organic substances and H_2O_2 and alleviate the stress (Eq. 1).

348 From Fig. 2b, c, d, the breakthrough points of all peroxidase enzymes were recorded since
349 day 25th. Concentration of enzymes in the root shifted steadily and ACT concentration in
350 effluent dropped below 0.4 µg/L. One indicated that all peroxidase enzymes started involving
351 in reducing ACT stress to plant. The root produced enzymes increasingly to degrade H_2O_2
352 and pollutants. The ACT concentration (10 mg/L) was substantial for the root; then it
353 translocated part of ACT to the shoot (Huber et al., 2009). Among enzymes, their
354 concentrations in plant ranged differently. Concentrations of SP enzyme reached to 0.3 unit/g
355 FW while concentrations of IP enzyme expanded to 0.2 unit/ g FW. For CP enzyme, the
356 concentrations varied from 0.01 to 0.16 unit/g FW. We speculated that the concentration of
357 SP enzyme was higher than the others because it presented in plant cell as a soluble form.
358 Hence, it was more dynamic than the bound enzymes such as IP and CP.

359 [Insert Fig. 2]

360 The hydroxyl radical group is the most striking agent that breaks the aromatic structure of
361 ACT, compared with CO_3^-, ClO^- and ferrate (VI) (Phong Vo et al., 2019). It targets the ACT
362 molecules by the normal and ipso mechanisms, following by a series of oxidation processes.

Thus, it liberates the ring core and cleaves the phenol-acetamido bond (Fig. 3). However, the hydroxyl radical group catalyzed by enzymes prefers invasing the acetamido moiety than the aromatic ring (Phong Vo et al., 2019). Accordingly, the phenol-acetamido bond was hydrolyzed to form p-aminophenol and acetic acid. Afterward, *p*-aminophenol was oxidized by the $^{\bullet}OH$ radicals forming numerous by-products.

[Insert Fig. 3]

For plant, the documented mechanism was also to form hydroxylated intermediates, glucose conjugate, cysteine conjugate of micro-pollutants (Huber et al., 2009; Huber et al., 2012). Peroxidase was oxidized by two electrons of H_2O_2 creating intermediates. The intermediates continually oxidized micro-pollutants to metabolites. Then the intermediates were reduced back to peroxidase enzyme (Zhang and Geißen, 2010). Based on those evidences, we proposed the ACT degradation mechanism in plant expressed as below:

$$H_2O_2 + ACT \rightarrow \text{3-hydroxyl-acetaminophen} + \text{N-acetyl-1,4-benzoquinone imine} +$$

$$\text{acetaminophen-glucoside} + \text{acetaminophen-glutathione} + \text{cysteine conjugate} + H_2O \quad \text{(Eq. 7)}$$

Among the by-products, N-acetyl-1,4-benzoquinone imine was a toxic one. It was detoxified by glutathione S-transferase pi 1 enzyme or reduced to ACT by NAD(P)H dehydrogenase enzyme (Hwang et al., 2015). Other by-products are non-toxic.

Micro-pollutants uptaken by the plant were reported previously; however, the mechanism of the enzymes in detoxification remains unclear. Up to date, enzymes occupied two functions: catalysts and oxidizers. Some enzymes could break down the aromatic structures, esters and nitrile bonds of micro-pollutants' molecules (Ufarté et al., 2015). Examples are laccase and glycosyltransferase enzymes. In cucumber plant, glycosyltransferase enzyme could oxidize ACT (Bartha et al., 2014). Some peroxidase enzymes, such as horseradish and lignin peroxidase, degrade diclofenac to diclofenac-2,5-Iminoquinone by catalysing H_2O_2 (Huber et

al., 2016). Still, peroxidase enzymes removed pesticide 2,4-dichlorophenol (Agostini et al., 2003). This study remains inconclusive as to whether the peroxidase enzymes could oxidize ACT directly. The detail mechanism still challenging and needed in-depth researches.

3.5 Correlations of enzymes and pollutants concentrations in CW effluent

The effluent concentrations of ACT, COD, NO_3^--N and TP were modelled following the enzymes concentrations. Firstly, the R^2 values of those models were calculated via Lindeman, Merenda and Gold's analysis (Table 3). The significant model should have R^2 values higher than 50%. Accordingly, the R^2 values of COD, NO_3^--N and TP models were 40-50% and they were insignificant for establishing models. The R^2 value of ACT model was around 80% that would be satisfactorily significant. For this ACT model, the Lindeman, Merenda and Gold analysis demonstrated that SP enzyme in the shoot and root and CP enzyme in the shoot contributed dominantly in the total R^2. Each of those variables contributed at least 15%. Other variables joined below 10% and would not be picked for constructing the model.

[Insert Table 3]

After determining the significant variables, the concentrations of ACT in effluent were modelled by three multivariable regression methods of "all possible subsets", "forward selection" and "backward elimination". As a result, only three variables were picked so that those methods exhibited similar outcomes (Eq. 8).

$$C_{ACT.E} = 0.089 - 1.744*C_{SP.R} + 9.802*C_{SP.S} - 84.487*C_{CP.S} \quad \text{(Eq. 8)}$$

(R^2=68.3%, p=0.01, F=6.46>3)

Comparing the coefficiencies of Eq. 8, the CP enzyme likely react the most actively in ACT removal, rather than the SP enzyme. One unit change in CP enzyme concentration was 10 to 50-fold larger than SP enzyme. Although the SP enzyme was generated more than the CP, its reactivity against ACT was much less. Depending on the interaction with cell wall,

411 peroxidase enzymes presented as soluble, ionically bound and covalently bound. Those
412 peroxidase enzymes reacted to organic compounds selectively (Kärkönen and Kuchitsu,
413 2015). For example, coniferyl and *p*-coumaryl alcohols could be oxidized by both anionic
414 and cationic peroxidase enzyme while sinapyl alcohols was oxidized a subgroup of cationic
415 peroxidase enzyme (Martínez-Rubio et al., 2018). Certain perixodase enzymes could oxidize
416 selective organic compounds thanks to steric hindrances at the substrates' binding site. For
417 this case, IP enzyme was probably not a catalyst for ACT degradation.

418 The characteristic of soluble peroxidase on ACT degradation is clear; however, the one of
419 ionical and covalent peroxidase is still on debate as those two peroxidase enzymes bind on
420 cell wall similarly. Recent finding indicates the biochemical function of ionical and covalent
421 peroxidase differ. Several reasons explain for their difference but the two important ones
422 including: molecular mass of enzyme and affinity to H_2O_2 (Hadzi-Taskovic Sukalovic et al.,
423 2015). For molecular mass, the value of ionical peroxidase marks 45 kDa while the one of
424 covalent peroxidase ranges 30, 40, 45 and 50 kDa. The fluctuating molecules size of covalent
425 peroxidase constitutes a complex structure between ACT and cell wall. Also, covalent
426 peroxidase attracts H_2O_2 more than ionical peroxidase which can lead to the more ACT
427 degradation efficiency. The covalent binding of covalent peroxidase increases affinity to
428 H_2O_2 and conditions H_2O_2 participating in the degradation cycle (Eq.1 - 3). For those reasons,
429 covalent peroxidase is more preferable involving in ACT degradation, together soluble
430 peroxidase, rather than ionical peroxidase.

431 4. Practical applications

432 This work exhibits the advance of vertical flow CW and peroxidase enzymes for hospital
433 wastewater removal and tracking. Those results tailor the practical applications in developing
434 countries. For those countries, CWs are prerequisite to reduce economic burden as capital and

operation costs are low. Capital cost for CW varies from 82 to 225 USD/m^3 wastewater, whereas it takes 246 – 657 USD/m^3 wastewater for a conventional treatment plant (Zhang et al., 2014b). In Vietnam, land is spacious that suitable for a low-cost CW treatment system. It is feasible for hospital wastewater treatment in Ho Chi Minh city - Vietnam - and suburban regions (Nguyen et al., 2019). The CWs can be co-functioned as wastewater treatment system and landscape decoration to increase green space and cut off capital cost, like the butterfly CW in Thailand (Brix et al., 2011). Not only for treating hospital wastewater, vertical flow CW is also useful for removing other micro-pollutants wastewater such as industrial, agricultural and aquaculture wastewater. The vertical flow CW can tolerate to high-dose micro-pollutants. Effluent would comply with wastewater discharge standards in Thailand and Vietnam. For the case, shrimp farm wastewater with antibiotics (Can Gio, Vietnam) is a viable target for vertical flow CW (Pham et al., 2018). In industrial and cattle wastewater, it contains nutrient and micro-pollutants in urine and faeces that also effectively removed and recovered 80% N by CW (Libralato et al., 2012).

Monitoring micro-pollutants is costly and using enzymes is an alternative for developing countries. One micro-pollutants sample costs at least hundred US dollar (Testamerica Laboratories, 2015) whereas analysing enzymes by regular chemicals is much cheaper. The micro-pollutants analysis requires facilities and skill to develop standard methods. In turn, enzyme analysis only needs spectrophotometer and standard chemicals. To deploy the concept in practice, more micro-pollutants and enzymes, such as laccase, superoxide dismutase, need in-depth studies.

5. Conclusion

The vertical flow CW effectively removed ACT and nutrients from hospital wastewater treatment. The peroxidase enzymes of *S. validus* planted in CW were feasible for monitoring

459 ACT. It could track ACT concentration in the CW effluent for pollution control. This novel
460 concept helped to reduce an extensive cost of micro-pollutants analytical facilities. Thus, for
461 full-scale application, the studied data is a reference for designing micropollutants phyto-
462 remediation process and monitoring. Similar micro-pollutants can be monitored by different
463 plant species and enzymes. Still, the mechanism of the enzyme and micropollutants' reaction
464 needs in-depth studies.

465

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470 of NATS lab staff is duly appreciated.

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655

656

657 List of Tables

658 Table 1. ACT removal of various CWs

	Type of CW	Influent ACT concentration	Removal efficiency (%)	Hydraulic retention time	Plant species	Media bed	Wastewater matrix	Reference
1	Subsurface vertical flow	10 mg/L	3.5 to 6 log	5 d	*S. validus*	Sand and gravel	Hospital wastewater	This study
2	Subsurface vertical flow	4.365 µg/L	0 - 100	3.5 d	*Salix alba*, *Iris pseudacorus*, *Juncus effusus*, *Callitriche palustris* and *Carex caryophyllea*	Mud and soil	Municipal wastewater	Nuel et al. (2018)
3	Vertical flow	Below 189 ng/L	Below detection limit	95 mm/d	*Phragmites australis*	Sand and gravel	Urban wastewater	Ávila et al. (2014)
4	Horizontal subsurface flow	273 ± 158.5 ng/L	20 - 69	0.58 d	*P. australis*	Steel slag and gravel	Urban wastewater	Petrie et al. (2018)
5	Horizontal subsurface flow	350 - 180000 ng/L	86.2 - 99.6	6.3 - 11.6 d	*P. australis* and *P. arundinacea*	Gravel	Raw sewage	Vymazal et al. (2017)
6	Horizontal subsurface	701-4938 ng/L	58.1	22.75 d	*Typha angustifolia*,	Gravel	Landfill leachate	Yi et al. (2017)

	flow				*Chrysopogon zizanioides* and *Cyperus papyrus*			
7	Horizontal subsurface flow	35 µg/L	95	3.5 d	*P. australis*	Gravel	Urban wastewater	Ávila et al. (2013)
8	Horizontal subsurface flow	30 ng/L	45	1 d	*P. australis*	Gravel	Municipal wastewater	Verlicchi et al. (2013)
9	Horizontal subsurface flow	750 ng/L	51.7 - 99	1.49 - 1.53 d	*P. australis* and *T. latifolia*	Soil, stone and gravel	Raw wastewater	Ranieri et al. (2011)

659

660

661 Table 2. Removal of other pollutants by the vertical flow CW

Parameters (mg/L)	Influent concentration (*n = 4*)	Effluent concentration (*n = 13*)	Removal efficiency (%)
TS	5745.0 ± 1076.8	6297.9 ± 1294.7	-
TSS	500 ± 236.8	82.8 ± 46.9	81.1 ± 2.8
TDS	5378.3 ± 1191.5	6115 ± 1269.3	-
NH_4-N	25.0 ± 6.4	1.3 ± 2.3	94.8 ± 11.9
TKN	36.6 ± 12.6	12.7 ± 6.9	65.3 ± 11.1
$NO_{3\ N}^{-}$	1.0 ± 0.6	12.2 ± 8.1	-
COD	52.7 ± 164.1	42.1 ± 23.8	88.1 ± 33.1
TN	42.3 ± 15.5	32.8 ± 20.3	22.5 ± 31.9
TP	7.9 ± 4.3	2.6 ± 0.8	67.1 ± 15.6

662

663

664 Table 3. Relative importance of variables in ACT, COD, TN and TP equations

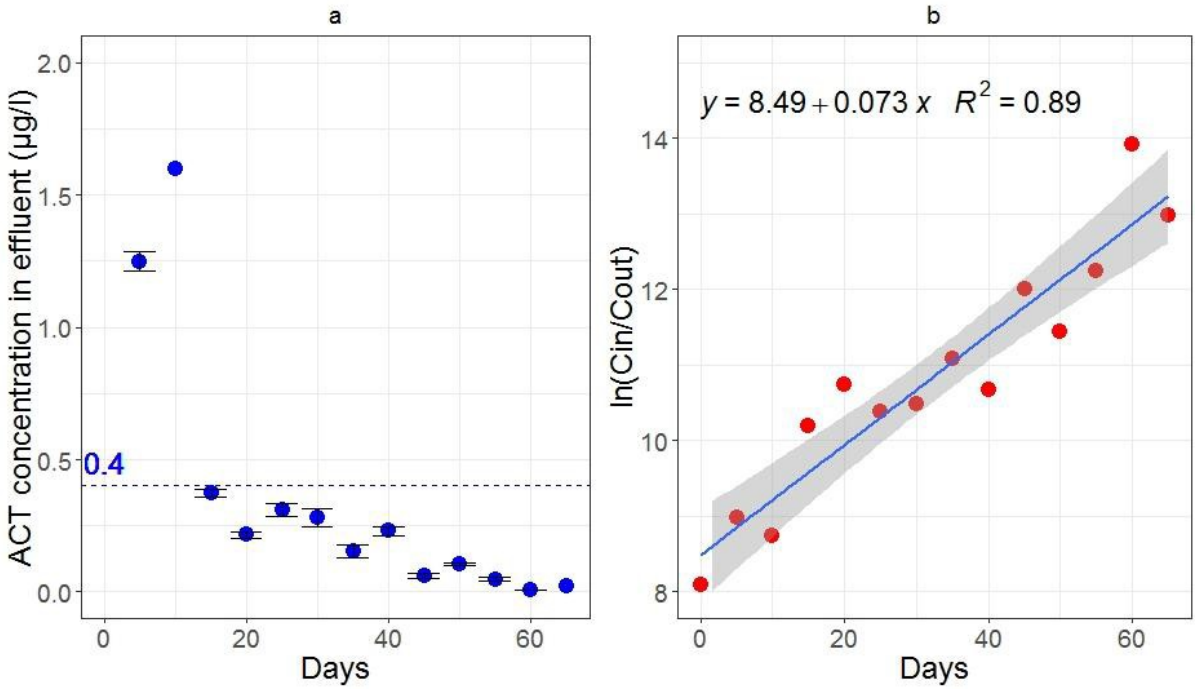
Relative importance (%)	$C_{H2O2.R}$	$C_{H2O2.S}$	$C_{SP.R}$	$C_{SP.S}$	$C_{CP.R}$	$C_{CP.S}$	$C_{IP.R}$	$C_{IP.S}$	Total R^2
ACT.E	5.9	4.2	**14.3**	**23.7**	6.1	**15.7**	3.4	6.1	79.4
COD.E	3.0	1.0	4.0	16.5	7.3	3.3	0.9	7.9	43.9
NO_3^--N.E	2.5	1.6	1.4	23.7	3.3	3.1	15.8	0.1	52.5
TP.E	4.8	2.4	0.1	10.4	1.0	3.4	1.6	18.6	42.9

665 Bold is the picked variables for constructing models

666

667

668 List of Figures



669

670 Fig.1. ACT concentration in CW effluent (a), kinetic of ACT removal in CW (b)

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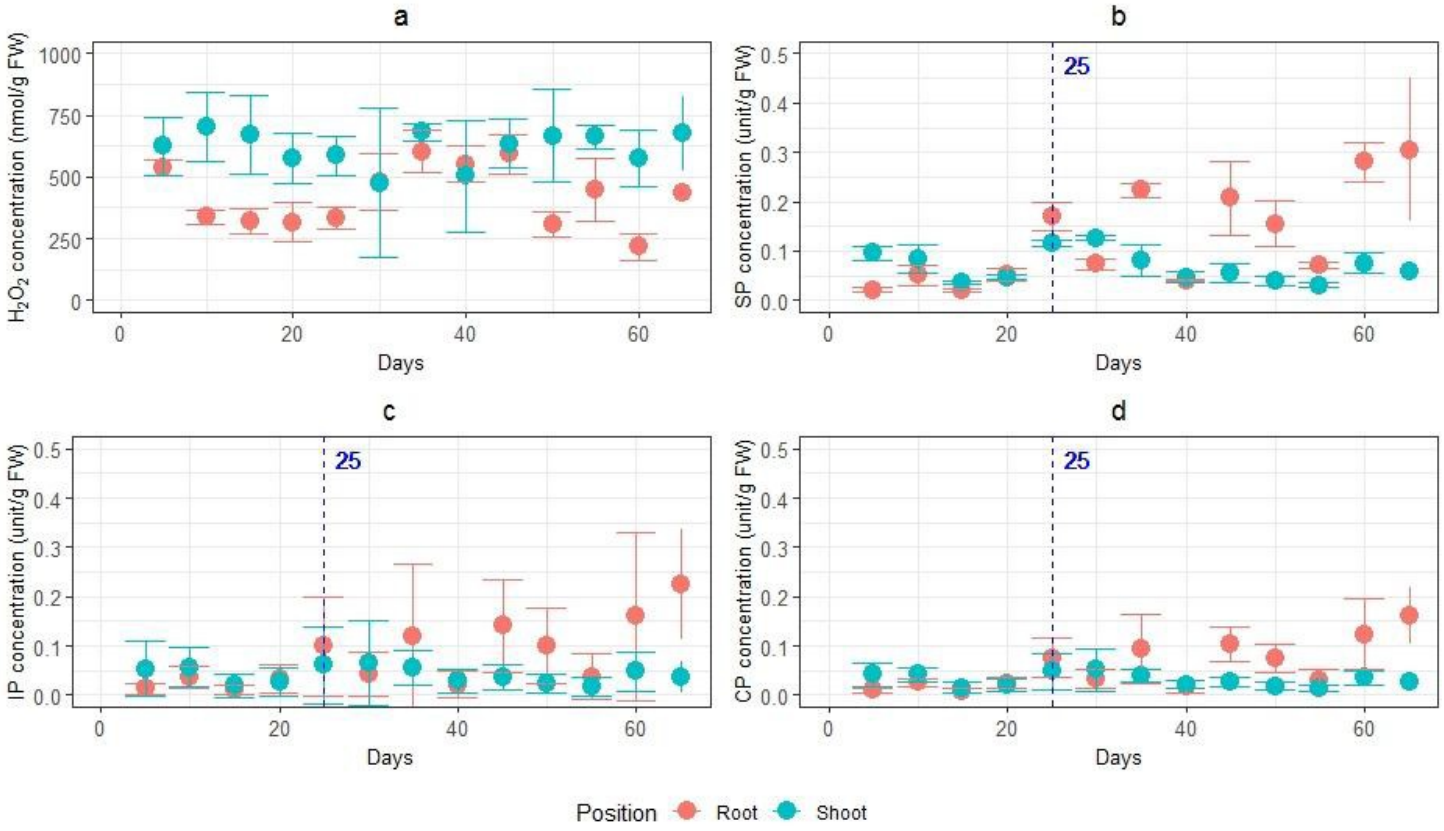

673

674 Fig. 2. Concentrations of H_2O_2 (a), SP enzyme (b), IP enzyme (c) and CP enzyme (d) in shoot and root of *Scirpus validus* (n=3). $p<0.05$ presents

675 the significant difference of enzymes concentration in shoot and root.

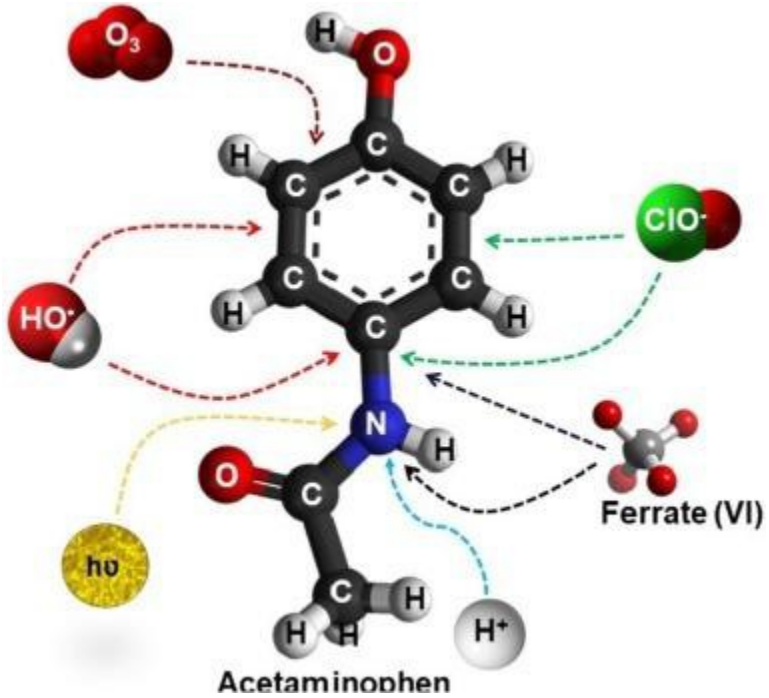

Fig. 3. Oxidative agents strike on bonding of ACT molecule. Retrieved from Phong Vo et al. (2019).