**Effects of tidal flushing regimes on mangrove roots receiving
wastewater contaminated with PAHs and PBDEs**

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Abstract

The effects of tidal flushing regimes on roots of wastewater-stressed mangrove plants were unknown. The present study showed that patterns of radial oxygen loss (ROL) in two mangrove species, *Excoecaria agallocha* and *Kandelia obovata*, receiving wastewater containing polycyclic aromatic hydrocarbons (PAHs) and polybrominated diphenyl ethers (PBDEs), were not affected by tidal flushing regimes. However, more ROL was induced under less frequent tidal flushing regimes, and produced along a root of *K. obovata* than that of *E. agallocha*. The iron (Fe) plaque formed on roots were negatively correlated to ROL, suggesting that Fe plaque functioned as a 'barrier' to prevent ROL, with stronger effects under less frequent tidal flushing regimes and in *K. obovata* with weaker outer layers. These results revealed that PAHs- and PBDEs-stressed *K. obovata* was more sensitive to tidal flushing regimes than *E. agallocha*, and long-term exposure would lead to root damage and even plant death.

Key words: Fe plaque, mangrove plants, polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers, radial oxygen loss

43 **1 Introduction**

44 Mangrove wetlands distributed along coastal areas are close to human activities
45 and are subject to anthropogenic contamination. Polycyclic aromatic hydrocarbons
46 (PAHs) and polybromodiphenyl ethers (PBDEs) are two types of persistent organic
47 pollutants commonly found in industrial discharges and in environments. PAHs, toxic,
48 mutagenic and carcinogenic, are resistant to biodegradation, especially in anaerobic
49 sediments where serious accumulation of PAHs can lead to significant adverse health
50 effects (Kanaly and Harayama, 2010; Toyama et al., 2011). PBDEs used as additive
51 flame retardants in a wide range of industrial production are also highly lipophilic and
52 bio-magnified in organisms through food chains and food webs (Ikonomou et al.,
53 2002; Kelly et al., 2008). PBDEs cause various toxic effects to animals such as
54 developmental neurotoxicity and thyroid hormone disruption (Branchi et al., 2002;
55 Darnerud, 2003). It is common to find that mangrove plants continuously receive
56 anthropogenic PAHs and/or PBDEs from wastewater discharges, as these pollutants
57 have been detected in mangrove ecosystems around the world, such as Hong Kong
58 SAR of China (Ke et al., 2005; Li et al., 2009; Zhu et al., 2014), Brazil (Farias et al.,
59 2008), Mainland China (Cao et al., 2009), Indonesia (Dsikowitzky et al., 2011), India
60 (Binelli et al., 2008) and Senegal (Bodin et al., 2011).

61 Being intertidal, mangrove plants are subject to tidal flushing, leading to
62 alternating aerobic and anaerobic conditions, and different oxygen content in soil.
63 Mangrove plants had a large ability to release oxygen from root aerenchyma air

spaces to rhizosphere, a phenomenon called radial oxygen loss (ROL), and form iron (Fe) plaque on root surfaces (Pi et al., 2010a, b, 2011). Not only adapt to waterlogged soil, ROL and Fe plaque also protect wetland plants against toxicants and environmental stresses (Armstrong, 1979; Brix, 1997; Visser et al., 2000; Pi et al., 2009). The patterns and rates of ROL, as well as the concentration of Fe plaque formed on plant roots, were profoundly affected by tidal flushing regimes and pollutants (Moller and Sand-Jensen, 2008; Pi et al., 2010a, b, 2011). However, how tidal flushing affects ROL and Fe plaque in mangrove plants continuously receive toxic pollutants is still unknown.

In Hong Kong, mangrove wetlands are mostly inundated by incoming tides twice a day, with the largest tidal range at high spring tides of about 2.8 m (Tam and Wong, 2000). The mangrove plants distributed in different tidal positions from seaward to landward are exposed to different frequencies of tidal flooding, depending on their tidal zonation. *Kandelia obovata*, the most common mangrove plant species found in all tidal positions in Hong Kong, are subject to various tidal regimes with different frequencies, while the plants grown in seaward area such as *Aegiceras corniculatum* and *Acanthus ilicifolius* are often flooded twice a day, on the contrary, those grown at the very landward location such as *Excoecaria agallocha* and *Heritiera littoralis* are only flooded twice a month or even never flooded (Tam and Wong, 2000; Pi et al., 2009). It is logical to hypothesize the responses of stressed mangrove plants to tidal flushing regimes vary among plant species at different tidal positions.

85 The present study therefore aims to investigate the effects of tidal flushing regimes
86 on the growth, ROL, formation of Fe plaque, and relationships between ROL and Fe
87 plaque formation, of mangrove plants continuously received wastewater containing
88 different congeners of PAHs and PBDEs. Two mangrove plant species, namely
89 *Excoecaria agallocha* L. and *Kandelia obovata* Sheue, Liu & Yong, were selected,
90 because they distribute in different tidal positions and have different root structures
91 (Pi et al., 2009). Pi et al. also found that *K. obovata* had larger aerenchyma air spaces
92 to transport O₂ within root and was more tolerant to waterlogging, while *E. agallocha*
93 having stronger outer layers to protect its root against soil toxic pollutants was more
94 tolerant to wastewater (Pi et al., 2009). This is the first study comparing the root
95 responses of these two species, under the stress of mixed PAHs and PBDEs, to
96 different tidal flushing regimes.

97

98 **2 Materials and methods**

99 **2.1 Preparation of one-year old seedlings**

100 One year prior to this study, the propagules of *E. agallocha* and *K. obovata* were
101 collected from local mangrove swamps in Hong Kong, germinated and grown in
102 small pots with one propagule per pot. Each small pot had a dimension of 9.6 cm in
103 diameter and 12.2 cm in height (total volume was 882.6 cm³), and contained surface
104 soil collected from a typical mangrove swamp in Hong Kong with the soil properties
105 described by Pi et al. (Pi et al., 2010a). The pots were placed in a greenhouse with a

light intensity of $57.29 \mu\text{mol m}^{-2} \text{s}^{-1}$, average temperature and humidity of 27°C and 70%, respectively. Every morning, the pots were irrigated with tap water to ensure that the soil was wet but without any water flooded on soil surface and water was gradually evaporated during the day. During the one-year growing period, no nutrients or salts were added as propagules stored enough nutrients for germination and initial growth. Ye and Tam also concluded that nutrients in mangrove soil were sufficient for subsequent growth and the mangrove seedlings in Hong Kong grew better at low salinity (Ye and Tam, 2002). The one-year seedlings were healthy with green leaves.

115

116 **2.2 Experimental design**

A total of 48 pots, each with a dimension of 12.4 cm in diameter, 10.1 cm in height and total volume of 998.4 cm^3 , filled with the same mangrove soil as described above to a depth of 9 cm, were prepared. Half of the pots were planted with *E. agallocha* and the other half with *K. obovata*, with one seedling obtained from the above section per pot. The transplanted seedling was allowed to acclimate in the new pot condition and was irrigated with tap water every morning to keep the soil moist for one month. After acclimation, the pots having the same plant species were further divided into four groups with different tidal flushing regimes: (i) everyday flushing (T_e), (ii) one-day with tidal flushing and one-day without (T_o), (iii) two days with tidal flushing per month (on the 1st and 15th day, T_t) and (iv) no tidal flushing (control, T_n), with three

127 replicates per group. Every day, each pot was irrigated with artificial wastewater
128 containing three compounds of polycyclic aromatic hydrocarbons (PAHs), that is,
129 phenanthrene (Phe), pyrene (Pyr) and benzo(*a*)pyrene (BaP), and six congeners of
130 polybrominated diphenyl ethers (PBDEs), that is, BDE-47, -100, -99, -154, -153 and -
131 209 during the exposure period (at about 15:00). The concentrations of Phe, Pyr, BaP,
132 BDE-47, -100, -99, -154, -153 and -209 in the artificially prepared wastewater were
133 870.00, 135.90, 9.20, 10.94, 3.34, 16.86, 1.21, 1.56 and 18.77 $\mu\text{g L}^{-1}$, respectively.
134 The concentrations of PAHs and PBDEs in the artificial wastewater in the present
135 study were chosen based on the following considerations: 1) the water solubility of
136 each contaminant based on the Estimation Programs Interface (EPI) Suite TM
137 (developed by the US Environmental Protection Agency's Office of Pollution
138 Prevention and Toxics and Syracuse Research Corporation) estimated from Log Kow
139 at 25°C and fragments, as well as the real measurement in our greenhouse; 2) the
140 levels of PAHs and PBDEs in raw wastewater reported in previous studies ranged
141 from few ng L^{-1} to tens mg L^{-1} (Song et al., 2006; Peng et al., 2009; Sanchez-Avila et
142 al., 2009; Clarke et al., 2010); and 3) the levels of individual PAH and PBDE
143 compound used in present study were their respective highest water solubility
144 detected in our greenhouse condition, comparable to or ten times higher than those in
145 field applications, in order to assess the effects of tidal flushing regimes on mangrove
146 roots in heavily-polluted areas. The three PAHs (Phe, Pyr and BaP) were purchased
147 from Sigma–Aldrich (St. Louis, MO, USA) with purity higher than 96%, and the six

148 PBDE congeners (BDE-47, -100, -99, -154, -153 and -209) were purchased from
149 AccuStandard (New Haven, CT, USA) with purity higher than 99%. The daily
150 hydraulic loading (the volume of artificial wastewater irrigated to each pot in every
151 tide) was 80 mL which is equivalent to a hydraulic retention time of 5 days.

152 To simulate tidal flushing, the artificial seawater, prepared by dissolving
153 commercially available salts in tap water with a salinity of 15 parts per thousands (ppt)
154 was flooded to each pot at a water depth of 4 cm above soil surface on the tidal
155 flushing day (from 18:00 pm to 10:00 am of the next day, about 16 hours) to simulate
156 high tide, followed by 8 hours low tide by draining seawater from the pot and let the
157 soil exposed and dried out gradually. Seawater was reused during the study. The pots
158 were placed in the same greenhouse as described above and the experiment lasted
159 eight months. Three pots from each group were randomly retrieved at the end of 4-
160 and 8-months. The seedling was harvested, ROL, Fe plaque formation on root surface,
161 and biomass of leaf, stem and root were determined.

162

163 **2.3 Determination of ROL along a lateral root and biomass**

164 The rates of ROL at fixed distances behind the tip (0, 2, 4, 6 and 8 cm from the root
165 tip) of the intact lateral root, with lengths ranging from 8 to 12 cm and approximately
166 6 cm below root-shoot junction, were measured in an O₂-free root medium using the
167 cylindrical root-sleeving O₂ electrode (Armstrong et al., 1994), within 24 hours after
168 harvesting. The detailed procedures were described in Pi et al. (Pi et al., 2010a, b). In

169 brief, the intact root system was gently immersed into the well prepared O₂-free
170 solution containing 0.5 mM CaSO₄ and 5 mM KCl into agar-water medium (0.05%
171 w/v), while the other parts of the plant were in air. The section of the lateral root to be
172 measured was inserted through a cylindrical O₂ electrode, and the ‘diffusion current’
173 generated by the reduction of oxygen at the polarized platinum surface was recorded,
174 at least two hours after transferring the root into the O₂-free solution. All
175 measurements were made at the polarizing voltage of 1 mV. The rate of ROL along a
176 root was calculated based on the ‘diffusion current’ at each position measured and
177 their respective surface area.

178 After the measurement of ROL, the same lateral root was cut and used for the
179 determination of Fe plaque. The plants were then divided into leaf, stem and root
180 portions, oven-dried at 70°C for 48-72 hours to calculate the oven-dried biomass of
181 each portion. The plant dry biomass of each species was also measured at the
182 beginning of the experiment.

183

184 **2.4 Determination of Fe plaque formation on root surfaces**

185 The lateral root selected was extracted using the cold dithionite-citrate-bicarbonate
186 (DCB) extraction method (Taylor and Crowder, 1983). The detailed procedures were
187 described in Pi et al. (Pi et al., 2010b, 2011). In brief, each collected root section was
188 washed and agitated for three hours in a cold DCB solution consisting of 40 mL of
189 0.3 M sodium citrate (Na₃C₆H₅O₇•2H₂O), 5 mL of 1.0 M sodium bicarbonate

190 (NaHCO₃) and 3 g of sodium dithionite (Na₂S₂O₄). The wash was collected and the
191 resulting solution was made to a volume of 100 mL. Then 20 mL of the solution was
192 used for the measurement of Fe plaque. The concentrations of Feⁿ⁺ were measured
193 with Inductively-Coupled Plasma-Atomic Emission Spectrometry (ICP-AES,
194 PerkinElmer, USA), according to the standard method (Page et al., 1992).

195 All analytical procedures were subject to strict quality assurance and control. The
196 samples were analyzed within 24 hours after each harvesting. Standard addition
197 method was employed to ensure the accuracy and recovery of the analysis. Five
198 calibration standards (prepared using Trace Metals Standard obtained from
199 AccuStandard, USA), with concentrations of Feⁿ⁺ ranging from 0 to 10 µg mL⁻¹, was
200 used for the quantification of Feⁿ⁺ by ICP-AES. Control standard with Feⁿ⁺
201 concentration at 5 µg mL⁻¹ was analyzed for every four samples to ensure instrument
202 performance during analysis. Each sample was repeatedly analyzed for three times,
203 the standard deviation was lower than 1% and the detection limit was 0.18 ng mL⁻¹.
204 The washed roots were freeze-dried to obtain the dry weight. The concentration of Fe
205 plaque on root surface was calculated according to Taylor and Crowder as follow
206 (Taylor and Crowder, 1983):

207 [Fe plaque on root surface]=0.1591 × [Feⁿ⁺] in the extraction / root dry weight (mg
208 g⁻¹ root d.wt).

209

210 **2.5 Statistical analyses**

The mean and standard deviation values of the triplicates of each measurement in each group were calculated. A parametric analysis of covariance (ANCOVA), with time as the covariate, and species vs. tidal flushing regimes as the two factors, was used to test any differences in plant dry biomass of each individual part, rate of ROL from one section of a root and total rate of ROL along a root, as well as the formation of Fe plaque on root surface. A parametric independent sample *T*-test was conducted to test any difference between the two sampling times in each tidal flushing regime in each species. The linear regressions between Fe plaque formed (x) and total rate of ROL released (y) were calculated. The differences of the regression coefficients between more (Te, To) and less tidal regime (Tt, Tn) in each plant species were tested by a parametric independent sample *T*-test. All tests were carried out using the Statistical Package for Social Science (SPSS 16.0 for Windows, SPSS Inc, USA) following the principles described in Zar (Zar, 1999).

3 Results and Discussions

3.1 Plant growth

All mangrove plants continued to grow during the entire experimental period with continuous discharge of wastewater containing PAHs and PBDEs, but rates of growth were affected by plant species, time and tidal flushing regimes (Fig. 1 and Table S1). Statistical analyses of the ANCOVA test also showed that the dried biomass of leaf, stem and root varied significantly between two mangrove species, among tidal

flushing regimes and between two sampling times (Table 1). The production of leaf,
 stem and root biomass of *E. agallocha* was faster from 0- to 4-month, with monthly
 growth rates varied between 31.85 and 482.56%, while that in second four months (4-
 to 8-month) were lower, between 0 and 47.98%, dependent on plant parts and tidal
 regimes (Fig. 1 and Table S1). Among different plant parts, temporal changes of stem
 growth was most significant, with monthly growth rates of more than 200% in the
 first four months to less than 20% in the second four months. Similar pattern was also
 found in the growth of *K. obovata*, with more rapid growth from the beginning to 4-
 month (monthly growth rates of each plant part varied between 20.85 to 661.44%),
 but plant growth was inhibited from 4-month onward except stem (Fig. 1 and Table
 S1). Among three plant parts, root growth of *K. obovata* showed the most significant
 temporal changes, with more than 200% growth from 0- to 4-month but dropped to
 negative growth from 4- to 8-month. These results revealed that growth rates differed
 significantly among plant species and even between plant parts of the same species.
 The faster growth rate of mangrove plants in the 1st four months than that in the 2nd
 four months may be due to the accumulation of PAHs and PBDEs in soil caused by
 the continuous discharge of wastewater, as these contaminants were resistant to
 biodegradation (Kanaly and Harayama, 2010; Toyama et al., 2011). Although the
 PAHs in the soil could be utilized as the sole carbon source to support plant growth
 (Toyama et al., 2011), accumulation of excessive PAHs and PBDEs could cause
 significant adverse effects to organisms exposed to them (Branchi et al., 2002;

253 Darnerud, 2003; Kanaly and Harayama, 2010; Toyama et al., 2011).

254 Tidal regimes were significant in affecting plant growth but its effect depended on
255 plant species and time (Fig. 1, Table 1 and Table S1). The growth rate of the entire
256 plant of *E. agallocha* in the first four months increased with decreases in the
257 frequency of tidal regime except Tn (without any tidal flushing); however, the
258 opposite was found from 4- to 8-month with faster growth in Te and To treatments
259 with more frequent tidal flushing (Fig. 1). Different from *E. agallocha*, the total
260 biomass production of *K. obovata* decreased with the frequency of tidal regime
261 throughout the whole experimental period (Fig. 1). Previous studies also found that
262 the effects of tidal flushing on growth of these two plant species in freshwater without
263 any contamination were different, with *K. obovata* showing positive response to tidal
264 flushing (Cheng et al., 2015), but *E. agallocha* was not affected by tidal regimes (Pi
265 et al., 2010a, b). The higher tolerance of *K. obovata* to waterlogging was related to its
266 larger root aerenchyma air spaces for transporting O₂ from aerial plant parts to root
267 tips (Pi et al., 2009). However, the present results on growth responses to
268 waterlogging were somewhat deviated from the study by Pi et al. (Pi et al., 2009),
269 variations in root growth rates due to tidal regimes with more flushing frequency
270 were larger in *K. obovata* (decreasing from 661% at 4-month to -10% at 8-month
271 with the highest rates in Te and To) than that of *E. agallocha* throughout the whole
272 experiment (Fig. 1 and Table S1), indicating *K. obovata* was more sensitive to more
273 frequent tidal flushing than *E. agallocha*. The continuous discharge of wastewater

contaminated with PAHs and PBDEs posed pollution stress to plant growth and such stress was more serious when plants were grown in waterlogged soils than that without waterlogging. Waterlogging created more anaerobic conditions in soil leading to the accumulation of toxic pollutants (Ponnamperuma, 1984; Colmer, 2003; Pi et al., 2010b). The “carrier” effects of water to transport pollutants from soil to plants also increased the exposure potential of plant roots to soil pollutants (Colmer, 2003; Pi et al., 2010a). Pi et al. found that *E. agallocha* developed much stronger root outer layers (epidermis and hypodermis) to protect its roots against soil toxic pollutants than *K. obovata* (Pi et al., 2009), suggesting that *E. agallocha* might be more tolerant to toxic pollutants. Among three plant parts, roots appeared to be most sensitive to soil contaminants (Pi et al., 2010a). Therefore, although *K. obovata* had a higher tolerance to waterlogging than *E. agallocha* in fresh water conditions (Pi et al., 2009), the weaker root protective outer layers in *K. obovata* made it more sensitive to tidal regimes under PAHs and PBDEs stresses. The significantly lower monthly growth rates of *K. obovata* from 4- to 8- month than in the first four months (from 0- to 4-month) but such temporal difference was relatively smaller in *E. agallocha* (Fig. 1 and Table S1) further supported our finding that the latter species was more tolerant to two stresses, waterlogging and pollution.

3.2 Rates and patterns of radial oxygen loss (ROL) from roots

The rates of ROL along a lateral root, similar to plant growth, varied significantly

295 between mangrove species, tidal flushing regimes and sampling times (Fig. 2 and
296 Table 1). The rates also varied among different sections along a root (Fig. 2). The
297 rates of ROL from roots of *E. agallocha* and *K. obovata* were the highest in the root
298 tip and declined in the basal and mature zones in seedlings collected from both 4- and
299 8-month. This indicated that all roots had the same ‘tight barrier’ pattern to ROL,
300 irrespective to species, sampling time and tidal regimes. Tight barrier ROL is an
301 important adaptation strategy for mangrove plants to flooding (Liu et al., 2009b; Pi et
302 al., 2009). However, higher values in the root tip were found under less frequent tidal
303 flushing regimes (Tt and Tn) than those under the more frequent tidal flushing
304 regimes, Te and To (Fig. 2). The differences of ROL rates from mature zone, basal
305 zone and root tip of the seedlings subject to Tt and Tn were also larger than Te and To.

306 Not only the rates of ROL along a root, the total rates of ROL also significantly
307 affected by mangrove species, tidal flushing regimes and sampling times (Fig. 3 and
308 Table 1). The total rates of ROL decreased from 4- to 8-month in both plant species
309 under all tidal regimes, probably due to the prolonged exposure of wastewater
310 contaminated with PAHs and PBDEs which became toxic, as evidenced by the lower
311 biomass production in the second four months than the first four months (Fig. 1,
312 Table S1), especially *K. obovata*. The toxic and suppression effects of PAHs
313 compounds and PBDEs congeners to mangrove plants were reported (Liu et al.,
314 2009a; Huang et al., 2013; Wang et al., 2014). Similarly, ROL in mangrove seedlings
315 also decreased significantly, especially in apical root zones, when stressed by copper,

316 zinc and lead, due to the inhibitory effects of heavy metals on growth, alteration of
317 root permeability and development of aerenchyma tissues (Liu et al., 2009b; Pi et al.,
318 2009; Cheng et al., 2012). Not only mangroves, ROL in various emergent wetland
319 plants was also significantly reduced by the irrigation of strong wastewater containing
320 high concentration of zinc, due to damages to root tissues such as aerenchyma and
321 root porosity by zinc in wastewater (Mei et al., 2014). Jackson et al. reported that
322 wastewater was an environmental stressor changing root properties of in rice (Jackson
323 et al., 1985). Phytotoxins such as organic acids and sulphide also decreased ROL
324 from rice and reeds by altering their root anatomic features (Armstrong and
325 Armstrong, 2005).

326 Additionally to pollutants, wetland plants change the amount of ROL with time,
327 and temporal variations may be different among species and environmental
328 conditions. The amount of ROL in rice changed during growth, with dramatic
329 increases from tillering to ear emergence and then decreased at the grain-filling stages
330 (Wang et al., 2013). In the present study, more O₂ was released from roots of both *E.*
331 *agallocha* and *K. obovata* under less frequent tidal flushing regimes (Tt and Tn) than
332 the two regimes with more frequent tidal flushing (Te and To), the differences in total
333 ROL rates were more obvious at the first four month than that at the second four
334 months and the extent of decrease was larger in Tt and Tn (Fig. 3). Between the two
335 plant species, more O₂ was released from the root of *K. obovata* than that of *E.*
336 *agallocha*, irrespective to sampling time and tidal regimes. The amount of ROL from

roots was found to increase when there was more O₂ demand in immediate root surroundings (Armstrong, 1994; Pi et al., 2010a). ROL is generally known as an adaptation of wetland plants to waterlogging by creating an oxidizing environment, with more ROL under more anaerobic conditions (Armstrong and Armstrong, 2005). However, opposite results were recorded in the present study that the total rates of ROL from roots, same as the changes along the entire root, were significantly lower in Te and To than Tt and Tn treatments (Figs. 2 and 3). This indicated that the pots subject to more frequent tidal flushing had less ROL even though the plants were under longer duration of waterlogging and should have higher O₂ demand. The low ROL result may be attributed to the formation of Fe plaque on root surfaces, which was suggested to function as the ‘barrier’ to prevent ROL according to previous researches (Moller and Sand-Jensen, 2008; Pi et al., 2010b).

3.3 Formation of Fe plaque on root surface and relationships with ROL

More Fe plaque formation on root surfaces was found in both *E. agallocha* and *K. obovata* subject to more frequent tidal flushing (Te and To) than Tt and Tn at the two sampling times (Fig. 4 and Table 1). The concentrations of Fe plaque also increased significantly from 4- to 8-month in both plant species, indicating that the longer the duration of the plants receiving wastewater, the more the Fe plaque formed. The formation of Fe plaque could protect plants against waterlogging and increased their survival in anoxic environments (Armstrong, 1971). More Fe plaque formation was

358 induced in mangrove roots receiving wastewater and heavy metals to immobilize the
359 pollutants thus protecting plants against their toxicity (Pi et al., 2011; Mei et al.,
360 2012). Plant species with more Fe plaque formation on root surfaces were more
361 tolerant and more preferable in constructed wetlands for wastewater treatment (Mei et
362 al., 2014). When compared *E. agallocha* and *K. obovata*, more Fe plaque was formed
363 on root surfaces of the former species under tidal regimes with more flushing
364 frequency (Te and To), but the difference was not significant under less frequent tidal
365 flushing regimes (Tt and Tn). This indicated that *E. agallocha* was more tolerant to
366 wastewater stress and waterlogging than *K. obovata*.

367 Significant negative relationships were found between the formation of Fe plaque
368 on root surfaces and the total rate of ROL along a root during the 8-month irrigation
369 with wastewater containing PAHs and PBDEs under all tidal treatments in both plant
370 species (Fig. 5 and Table S2). Similar negative relationship between Fe plaque
371 formation and ROL around rhizosphere of *Lobelia dortmanna* was also reported by
372 MØller and Sand-Jensen that Fe plaque acted as a ‘barrier’ preventing oxygen
373 released from the root leading to lower ROL (Moller and Sand-Jensen, 2008). In
374 constructed wetlands, wastewater addition significantly decreased ROL but induced
375 more Fe plaque formation in various wetland plants, and these two parameters
376 exhibited negative correlations under wastewater stress (Pi et al., 2010b; Mei et al.,
377 2014; Yang et al., 2014). On the contrary, positive relationships were found in rice
378 that more ROL released from the root led to a higher concentration of Fe plaque

379 (Armstrong, 1967). Otte et al. concluded that ROL contributed to the formation of Fe
380 plaque on root surfaces in most wetland plants under waterlogged condition (Otte et
381 al., 1991). The interaction between the rate of ROL and the concentration of Fe
382 plaque formation in mangrove plants changed with time and wastewater treatment,
383 with positive correlations in the control (without wastewater) and at the initial phase
384 of the experiment, but changed to negative correlations afterwards and also in plants
385 receiving strong wastewater (Pi et al., 2010b). Their study, together with the present
386 findings on *E. agallocha* and *K. obovata* (Fig. 5 and Table S2), indicated that the
387 formation of excess Fe plaque on root surfaces would function as a ‘barrier’ to
388 prevent ROL in mangrove plants.

389 In both mangrove species at the same sampling time, regression relationships
390 between the two treatments with more frequent tidal flushing (Te and To) followed
391 the same trend which could be grouped together, while the two less frequent flushing
392 treatments (Tt and Tn) formed another group (Fig. 5). The regression coefficient (B)
393 between Fe plaque and ROL in the second group (Tt and Tn) were significantly
394 higher than that in Te and To group (Table S2). Between the two plant species, B
395 values of *K. obovata* were higher than that of *E. agallocha*, indicating the ‘barrier’
396 effect of Fe plaque on root ROL was more obvious in *K. obovata* (Table S2). This
397 may be related to the weaker outer layers in roots of *K. obovata* than that in *E.*
398 *agallocha*, thus required more barrier to prevent ROL (Pi et al., 2009).

399 The amount of Fe plaque formed on root surfaces was largely affected by the redox

potential of soil (*Eh* value) relating to the concentrations of Fe^{2+} in soil (Mendelssohn et al., 1995). It is because only soluble Fe^{2+} could be drawn by the transpiration stream to reach plant root surfaces, spontaneously oxidized by root released O_2 , and then precipitated as Fe plaque (St-Cyr and Crowder, 1989; Chen et al., 2008). The duration and frequency of tidal flushing could affect the mobility of Fe^{2+} in soil and the amount of O_2 around root surface (ROL) to oxidize Fe^{2+} (St-Cyr et al., 1993). It has been shown that submerged rice formed more Fe plaque on roots than the plants grown in aerobic soil, as the decrease of soil redox potential during flooding would lead to an increase of Fe^{2+} concentration in soil and larger amounts of dissolved Fe^{2+} would be moved to rhizosphere and oxidized by ROL to form Fe plaque (Liang et al., 2006; Chen et al., 2008). In aerobic soils, the mobility of Fe^{2+} may have decreased because of its precipitation and adsorption onto the bulk soil (Kirk, 2004). Similarly, Mendelssohn et al. also found that the exchangeable Fe in soils increased significantly during flooding leading to more Fe plaque formed on root surfaces (Mendelssohn et al., 1995).

The relationship between Fe plaque formation and soil redox potential was elucidated in several studies, suggesting that the concentration of Fe plaque formed on root surfaces was negatively correlated with the *Eh* value of the soil (Mendelssohn et al., 1995; Pi et al., 2010b, 2011). Similar findings were also obtained in the present study that more Fe plaque was formed in plants subject to more frequent tidal flushing (from Tn and Tt to To and Te) and longer duration of wastewater discharge

(from 4- to 8-month) (Fig. 4). The Te- and To-treated soils had more negative redox potential, and the *Eh* values decreased significantly from 4- to 8-month in all treatments. The *Eh* values from 4- to 8-month in Te-, To-, Tt- and Tn-treated soil were -120.7 to -160.0 mV, -98.3 to -115.7 mV, 135.4 to 112.2 mV and 157.7 to 134.3, respectively.

4 Conclusions

The present study is the first time reporting ROL and Fe plaque formation on root surfaces of two mangrove plants, *K. obovata* and *E. agallocha*, continuously received wastewater containing PAHs and PBDEs, two types of very toxic and persistent organic pollutants. The amounts of ROL in both plant species were affected by the frequency of tidal flushing, with less ROL around the rhizosphere when plants were subject to more frequent tidal flushing (Te and To regimes). However, the pattern of ROL was not affected by the frequency of tidal flushing regimes, and all plants exhibited ‘tight barrier’, irrespective to species, tidal regimes and sampling times. Compared the two plant species, *K. obovata* had more ROL than *E. agallocha*, and their responses to tidal flushing regimes and treatment time were also different. Formation of Fe plaque on root surfaces was negatively correlated with ROL, suggesting that Fe plaque functioned as a ‘barrier’ to ROL, the more the Fe plaque formed, the less the O₂ released from roots. The increase in the frequency of tidal flushing regimes decreased the ‘barrier’ effects of Fe plaque on ROL. The ‘barrier’

effect of Fe plaque on ROL in *K. obovata* with weaker root outer layers was more significant than that in *E. agallocha* having stronger outer layers. These results revealed that tidal flushing regimes significantly affected mangrove roots, especially in *K. obovata*, the more sensitive species to tidal flushing, with root damages and even death when grown in waterlogged soils continuously receiving wastewater-borne PAHs and PBDEs.

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Figure legends

Fig. 1 Effect of tidal regimes on dry biomass of leaf, stem and root in mangrove plants receiving wastewater contaminated with PAHs and PBDEs at 4-month (4M) and 8-month (8M) of the experiment (0: initial biomass; Te: Everyday flushing, To: tidal flushing at alternative day, Tt: tidal flushing twice per month, Tn: No tidal flushing; mean of three replicates were shown)

Fig. 2 Effects of tidal regimes on patterns of radial oxygen loss (ROL) along a lateral root in mangrove plants receiving wastewater contaminated with PAHs and PBDEs at 4-month (4M) and 8-month (8M) of the experiment (Te: Everyday flushing, To: tidal flushing at alternative day, Tt: tidal flushing twice per month, Tn: No tidal flushing; mean and standard deviation of three replicates were shown)

Fig. 3 Effects of tidal regimes on total radial oxygen loss (ROL) along a lateral root in mangrove plants receiving wastewater contaminated with PAHs and PBDEs at 4-month (4M) and 8-month (8M) of the experiment (Te: Everyday flushing, To: tidal flushing at alternative day, Tt: tidal flushing twice per month, Tn: No tidal flushing; mean and standard deviation of three replicates were shown; different letters in each tidal flushing treatment indicated significant difference between 4 and 8 months at $p \leq 0.05$ according to independent sample *T*-test)

Fig. 4 Effects of tidal regime on formation of Fe plaque on root surface in mangrove plants receiving wastewater contaminated with PAHs and PBDEs at 4-month (4M) and 8-month (8M) of the experiment (Te: Everyday flushing, To: tidal flushing at alternative day, Tt: tidal flushing twice per month, Tn: No tidal flushing; mean and standard deviation of three replicates were shown; different letters in each tidal flushing treatment indicated significant difference between 4 and 8 months at $p \leq 0.05$ according to independent sample *T*-test)

Fig. 5 Relationship between the formation of Fe plaque on root surface and total ROL along a lateral root during the 8-month experiment (4M: 4-month, 8M: 8-month; Te: Everyday flushing, To: tidal flushing at alternative day, Tt: tidal flushing twice per month, Tn: No tidal flushing; $n = 12$; Significance of regression coefficient *B* and coefficient of regression determination R^2 are summarized in Table S2)

Fig. 1

(a) *E. agallocha*

(b) *K. obovata*

Fig. 2

E. agallocha

K. obovata

Fig. 3

E. agallocha

K. obovata

Fig. 4

(a) *E. agallocha*

(b) *K. obovata*

Fig. 5

Table 1 Statistical analyses of the ANCOVA test, with time as the covariate, and species vs. tidal flushing regimes as the two factors, showing effects of mangrove plant species, tidal flushing regimes and sampling time on the plant dried biomass, ROL from roots, and Fe plaque formed on root surface (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, NS: not significant)

Parameters		Sources of variations			
		Species (Sp)	Tidal flushing (Tf)	Sampling time (St)	Sp x Tf
Plant dried biomass	Leaf	123.85***	8.42***	6.96**	7.94***
	Stem	388.42***	7.50***	16.08***	6.46**
	Root	39.57***	3.31*	11.02**	0.26 ^{NS}
ROL from roots	Rates along a root	62.79***	59.79***	172.87***	2.06 ^{NS}
	Total rates	39.03***	31.69***	87.27***	0.40 ^{NS}
Fe plaque on root surface		59.54***	248.73***	48.58***	19.93***