Photodegradation and sorption govern tetracycline removal during wastewater treatment in algal ponds

Norvill, Zane N.[a, b]; Toledo-Cervantes, Alma[a]; Blanco, Saul[c]; Shilton, Andy[b]; Guieysse, Benoit[b]; Muñoz, Raul[a*]

[a] Department of Chemical Engineering and Environmental Technology, University of Valladolid, Dr. Mergelina s/n, Valladolid 47011, Spain

[b] School of Engineering and Advanced Technology, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand

[c] The Institute of the Environment, La Serna, 58, 24007 Leon, Spain

*Corresponding author: mutora@iq.uva.es

Abstract

The degradation of the antibiotic tetracycline, supplied at 100 μg L^{-1} in domestic wastewater, was studied in an outdoor, pilot scale, high rate algal pond (HRAP). Effective operation was demonstrated with the biomass concentration and the chemical oxygen demand removal efficiency averaging 1.2 ± 0.1 g_{TSS} L^{-1} and 80 ± 4%, respectively, across all operational periods. Tetracycline removal exceeded 93% and 99% when the HRAP was operated at hydraulic retention times of 4 and 7 days, respectively. Batch tests and pulse testing during HRAP operation repeatedly evidenced the significance of photodegradation as a removal mechanism. Sorption dominated tetracycline removal during the night, but accounted for less than 6% of the total pollutant removal based on sorbed tetracycline extracted from biomass. Overall, these results provide the first demonstration of efficient antibiotic removal, occurring mainly via indirect photodegradation, during relevant HRAP operation (low pollutant concentration, domestic wastewater and natural sunlight).

Keywords: Emerging pollutant; microalgae; raceway; photolysis; wastewater

1. Introduction

Antibiotics are emerging pollutants of particular concern due to their widespread use in human and animal medicine (González-Zorn and Escudero, 2012; Lupo et al., 2012). Total global antibiotic use in 2010 exceeded 70 billion standard units (i.e. tablets) for human consumption (Gelband et al., 2015), and over 63,000 tonnes were used for livestock production (Van Boeckel et al., 2015). Since 60-90% of the administered antibiotic dose is commonly excreted in urine/faeces, antibiotics are therefore ubiquitous in wastewater (Hirsch et al., 1999; Sarmah et al., 2006). Conventional biological wastewater treatment (WWT) technologies (e.g. activated sludge, nitrification/denitrification systems) are not designed to target antibiotic removal specifically, often leading to the release of antibiotics to the environment, depending on the specific antibiotic and WWT system (Leung et al., 2012; Michael et al., 2013; Zhang and Li, 2011; Norvill et al. 2016). This release is suspected to be contributing to the increase of antibiotic resistance, which is a major human health risk (Aminov, 2009; Daghrir and Drogui, 2013; Gullberg et al., 2011). There are also environmental risks associated with the uncontrolled discharge of antibiotics, as the presence of antibiotics can alter microbial ecology and their ecological functions (Aminov, 2009; Daghrir and Drogui, 2013; Ding and He, 2010).

Algal based WWT by high rate algal ponds (HRAPs) is an emerging technology receiving increasing interest due to its energy efficient operation and resource recovery potential via carbon and nutrient assimilation into biomass production. In HRAPs, the removal of macropollutants (C, N, P) and the biomass production potential are now relatively well-

characterized (Park et al., 2013, 2011; Posadas et al., 2015a; Sutherland et al., 2014), but for HRAP, and indeed all algal WWT, there is scarce research on the fate of emerging pollutants, especially antibiotics (de Godos et al., 2012; Hijosa-Valsero et al., 2010; Matamoros et al., 2015). As with activated sludge, algal WWT systems are not designed specifically for emerging pollutant removal, but these systems present a number of unique characteristics compared to conventional municipal WWT systems (e.g. activated sludge), which may enhance antibiotic removal (Norvill et al., 2016). In particular, HRAPs are i) operated at long hydraulic residence times (HRT; several days rather than hours) allowing time for antibiotic removal by mechanisms with slow kinetics; ii) designed with high surface-area-to-volume ratios leading to high sunlight exposure and potential antibiotic removal by photodegradation; while also enabling iii) the co-existence of phototrophic, chemoorganotrophic and chemolithotrophic metabolisms supporting a broader catabolic potential for antibiotic biodegradation; iv) the co-existence of autotrophic and heterotrophic microorganisms enhancing biomass productivity and thus a potentially high antibiotic removal capacity by sorption; and v) daily variations in dissolved oxygen concentration and pH levels as a result of photosythetic activity, which may affect antibiotic removal mechanisms by changing the redox conditions or antibiotic structure. Specific research is therefore required given the complexity and specificity of the removal mechanisms and parameters potentially involved.

TET, of which tens of thousands of tonnes are produced worldwide annually, was selected as the target antibiotic (Daghrir and Drogui, 2013). Recent research by de Godos et al. (2012) investigated the removal of tetracycline (TET) in lab-scale HRAPs and concluded

that photodegradation and sorption were the main TET removal mechanisms, with insignificant biodegradation observed. However, the relevance of this work was limited, as synthetic wastewater, artificial light and a mono-algal culture of *Chlorella vulgaris* was used in their experimental design. On the other hand, TET may be biodegraded by bacteria under nitrifying conditions (Song et al., 2015), and Dzomba et al. (2015) reported that TET was biodegraded by four axenic algae cultures of *Pseudokirchnerilla subcapitata*, *Selenastrum capricornutum*, *Haematoloccus pluvialis* and *Chlorella* sp.. Since mixed algal-bacterial cultures (typically encountered in outdoors HRAPs) can behave very differently to bacterial nitrifying sludge or mono-cultured algae (Subashchandrabose et al., 2013), specific research is therefore required to investigate TET removal in HRAPs.

In this context, the aim of this paper was to investigate the fate of tetracycline (TET) during outdoor continuous operation of a pilot HRAP treating real domestic wastewater. The influence of the presence of TET on the WWT performance of the HRAP was also investigated. Additionally, batch experiments and pulse TET tests in the pilot HRAP were carried out in order to identify the relative contribution of potential removal mechanisms (photodegradation, biodegradation, sorption, and hydrolysis).

2. Materials and Methods

2.1 Chemicals

Analytical grade TET (>98%) was purchased from Sigma Aldrich (Spain). HPLC-grade acetonitrile (ACN), formic acid (85%) and the rests of the chemicals (reagent grade) were purchased from PANREAC (Barcelona, Spain).

2.2 Batch experiments

TET removal was studied in 2.5 L 'batch reactors' (open-top plastic tanks magnetically stirred at 250 rpm, 185 mm surface diameter and 145 mm liquid depth) supplied with algal-bacterial biomass sourced from the pilot HRAP described in Section 2.3. The biomass was sourced from the HRAP during stable operation with TET-spiked influent. Therefore, the biomass was acclimatized to the presence of TET. TET was added to the cultivation broth from a 100 mg L^{-1} aqueous stock solution and the broth was vigorously mixed for 30 seconds before sampling. TET concentration, pH, photosynthetic active radiation (PAR), dissolved oxygen (DO) concentration and temperature were recorded at each sampling time. The total suspended solids (TSS) concentration was measured for each batch reactor at the end of the experiment. These batch experiments were conducted with initial TET concentrations of 2 mg L^{-1} for ease of analysis, with the lower-concentration tests in the pilot HRAP performed using the more time-consuming and expensive SPE analysis. Three separate experiments were conducted to elucidate the mechanisms underlying TET removal in algal-bacterial systems, as detailed below:

2.2.1 TET removal during full-day outdoor test

To elucidate the relative contribution of photodegradation, biodegradation, sorption and hydrolysis on TET removal in the presence of algal-bacterial biomass, TET removal was investigated in the presence of either active biomass or dead biomass (autoclaved at 121°C for 20 min) during full-day outdoor tests (diurnal light exposure). An additional batch reactor with TET in ultrapure (MQ) water was used as a control. The batch reactors were

positioned beside the pilot HRAP and therefore subjected to the same climatic conditions. Following TET addition (2 mg L^{-1} initial concentration), the batch reactors were monitored for 22 hours: twice in the first evening, twice the next morning before direct sunlight and every two hours throughout the day.

2.2.2 TET sorption to biomass at different TET concentrations

To investigate TET sorption under dark conditions, the batch reactors were filled with 2.25 L of active algal-bacterial biomass, a variable volume of TET stock solution required to set the different initial concentrations of TET (0.2, 0.5, 1, 2, 5, and 10 mg L^{-1}), and MQ to reach a final total volume of 2.5 L. The batch reactors were incubated at 20 ± 3 °C and TET concentration was monitored over 14 hours (0, 0.5, 1, 4, and 14 h). Biomass samples (40 mL) were taken after 4 hours and 14 h of incubation and subsequently extracted (see Section 2.4) to quantify the amount of TET sorbed onto biomass.

2.2.3 Influence of pH on TET removal

The effect of pH on TET removal was investigated in the presence of active algal-bacterial biomass under dark conditions at 20 ± 3°C. Three batch reactors were adjusted to pH 6, 8.5 and 10.5 before sampling, using 0.1 M HCl or 0.1 M NaOH solution. These pH values were chosen according to the pKa values of TET (the two upper pKa values are 7.8 and 9.6; Qiang and Adams, 2004). Although our pilot HRAP did not reach as high as pH 10.5 (see Section 3.2.1), the pH in other HRAPs can typically attain values of 11 during peak solar hours (Norvill et al., 2016). Two batch reactors, one with the same biomass but without pH

adjustment (pH 6.5-6.8) and the other filled with MQ water, served as controls. The batch reactors were monitored over 4 hours (0, 1, 2, and 4 h).

2.3 Continuous wastewater treatment in HRAP

The 180 L oval pilot HRAP was constructed in PVC (L×W×D = 1.7 m × 0.82 m × 0.25), with a central wall and mid-channel baffles at each end (Figure S1, Supporting Information). A paddle wheel (6 blades, 10.5 rpm) supported a mid-channel velocity of the cultivation broth of 0.2 m s^{-1} according to Park et al. (2011). The HRAP was located outdoors at the Department of Chemical Engineering and Environmental Technology of the University of Valladolid (Spain, Mediterranean climate). The HRAP was inoculated with 1.5 L of 23 g_{TSS} L^{-1} settled biomass taken from an indoor HRAP of similar design (Posadas et al., 2015b).

2.3.1 Continuous HRAP operation

Fresh domestic wastewater was collected every 1-2 days from a pilot-scale pre-treatment plant (1 mm rotary sieve followed by primary sedimentation) sourced from a municipal sewer located nearby the Department of Chemical Engineering and Environmental Technology at University of Valladolid, Spain. The pre-treated wastewater was stored at 4-6°C, mixed with a submergible pump and fed continuously into the HRAP using a peristaltic pump. The HRAP effluent overflowed to an 8 L clarifier, where settled biomass was removed twice weekly. Pond performance was monitored twice a week by measuring the concentration of TSS and VSS (volatile suspended solids) in the HRAP, and the influent and effluent concentrations of COD (chemical oxygen demand), TOC (total

organic carbon), TN (total nitrogen), dissolved nutrients (NO_3^-, NO_2^-, PO_4^{3-}), TSS and VSS. Daily influent and effluent flow rates were measured, and the daily net evaporation rates determined. pH, DO concentration and temperature in the cultivation broth were recorded online. The average sunlight irradiation during each operational stage is shown in Table 1.

The HRAP was operated at 7 d HRT for 52 days (Stage I) to establish a baseline HRAP performance. TET was then supplied at 100 µg TET L^{-1} in the influent wastewater for 35 days at 7 d HRT (Stage II). The influent wastewater flow rate was increased in Stage III to operate the HRAP at 4 d HRT under the same TET influent concentration for 17 days. The 4 d HRT was maintained during Stage IV for another 27 days in order to perform TET pulse tests as described in Section 2.3.2. The 100 µg TET L^{-1} supplied in the influent wastewater is a concentration typically found in wastewater from hospitals (Pena et al., 2010). During the pseudo-steady states of HRAP operation in Stages II and III (Table 1), the aqueous TET concentration was monitored both in the mornings and evenings at least twice weekly. Samples of algal-bacterial biomass (40 mL) were taken for analysis of sorbed TET concentrations twice during each pseudo-steady state.

2.3.2 TET pulse tests during continuous HRAP operation

Several TET 'pulse tests' – adding 180 mL of 100 mg TET L^{-1} to the pilot HRAP – were conducted during Stage IV in order to confirm the findings from the batch experiments (Section 2.2) using lower initial TET concentrations. Each TET addition therefore increased the TET concentration in the cultivation broth by 100 µg L^{-1}. No TET was added

to the influent wastewater during the pulse test, but the HRAP was fed with TET-spiked wastewater between pulse experiments to maintain TET acclimatization of the biomass.

The standard procedure for the TET pulse tests included two TET additions in a single day, to observe any differences between TET added during sunlight exposure and TET added at night. Aqueous TET concentrations were measured twice before each pulse experiment to establish a baseline. The first TET addition was administered in the morning (10:30-11:30 am) and aqueous TET concentrations in the HRAP were monitored regularly until 7:30 pm. At 7:30 pm a second TET addition was administered, and the aqueous TET concentrations in the HRAP were monitored every 30 min for 90 min after the second pulse, and then every 2 hours the following day from 8 am until at least 4 pm.

2.4 Analytical methods

TET was analyzed by HPLC-UV (Waters e2695) using a Kinetex Core-shell C-18 150 mm × 4.6 mm column (particle size: 2 µm; pore size: 100 Å) and a UHPLC C-18 guard column from Phenomenex (CA, U.S.A.). TET was eluted with 1 mL min^{-1} gradient flow composed of eluent A (0.1% formic acid in MQ water) and eluent B (0.1% formic acid in ACN). The initial gradient composition was 97% eluent A and 3% eluent B, followed by a linear gradient increase to 55% B by 4.5 min, then a gradient increase to 95% B over 0.2 min, and held constant at 95% B for 2.2 min. The eluent composition was then returned to 3% B and held constant at 3% B for 2 min to re-equilibrate the column before the next analysis (total run time 9 min). The column was maintained at 25°C. Standard injection volume was 50 µL. The quantification limit was 20 µg L^{-1}. Peak areas were detected at 360 nm (2998 UV-

vis PDA detector) and were analyzed using Empower 3 software. The retention time of TET was 4.4 ± 0.1 min. The TET concentrations henceforth reported are a summation of 4-epi-tetracycline (4epiTET; retention time 4.2 ± 0.1 min) and TET concentrations. 4-epiTET is an epimer of TET that exists in equilibrium with TET in water, has a similar absorption spectrum near 360 nm, and still exhibits antibiotic activity (McCormick et al., 1957). Aqueous samples from batch tests were filtered through nylon syringe filters (0.22 µm). To minimize interferences and buffer all samples to a uniform pH, 0.2 mL of 0.1 M citric acid solution and 0.01 mL of 5% w/w Na_2EDTA solution were added to the 1 mL sample in the HPLC vial (Yang et al., 2005). Samples were stored at -4°C for up to 1 week until HPLC-UV analysis was performed.

The amount of TET sorbed on biomass was quantified using an extraction procedure adapted from Anderson et al. (2005). For this purpose, a 40 mL biomass aliquot of known TSS concentration was centrifuged at 20,000 g for 5 min. The supernatant was then discarded and 10 mL solvent (1% formic acid, 25% ACN, 75% H_2O) was added to the pellet. The solvent/biomass sample was finally mixed under dark conditions (12-19 hours) at 350 rpm, filtered (0.22 µm, nylon) and analyzed by HPLC-UV.

Solid phase extraction (SPE) was used to quantify the aqueous TET concentration in HRAP samples. Based on the protocol described by Yang et al. (2005), HRAP samples were immediately filtered with a combined glass filter (0.7 µm) and mixed-cellulose-ester Millipore (0.45µm) filter. Then, one mL of 5% Na_2EDTA solution was added to each 100 mL filtrate sample. If SPE was not immediately performed, samples were stored frozen (-

4°C) for up to a week. SPE cartridges (Oasis HLB Plus Short Cartridges, 225 mg sorbent, 60 µm particle size) were activated by three consecutive washes with 3 mL of CH_3OH, 3 mL of 0.5 N HCl solution and 3 mL of MQ water. Immediately before extraction, 20 mL of 0.1 M citric acid solution was added to the sample, and the pH was adjusted to <3 with 0.5 N HCl solution. The acidified sample was dripped through the SPE cartridge at 5 mL min^{-1}, followed by a 3 mL MQ water rinse. Excess water was expelled by passing air through the cartridge. TET was then eluted using 2 mL of CH_3OH. The eluent was stored in HPLC vials at -4°C for no more than two weeks before HPLC-UV analysis. Samples were analyzed by HPLC-UV as described above, but using a 25 µL injection volume since the CH_3OH matrix adversely affected quantification above 30 µL injection volume. The quantification limit of TET using SPE-HPLC-UV was 2 µg L^{-1}.

Standard methods for the analysis of wastewater were used to quantify the concentration of COD, TSS, and VSS (Clesceri et al., 1998). The concentrations of TOC and TN were determined using a Shimadzu TOC-VCSH analyzer (Japan) equipped with a TNM-1 chemiluminescence module. NO_2^-, NO_3^- and PO_4^{-3} were analyzed by HPLC as described by Posadas et al. (2014). The morphological identification of microalgae was carried out by microscopic observations (OLYMPUS IX70, USA) using samples preserved with 5% Lugol's iodine according to *Phytoplankton Manual* (Sournia and Caspers, 1980). The determination of the carbon, hydrogen and nitrogen contents of the biomass was performed using a LECO CHNS-932, while the quantification of phosphorous and sulfur contents was carried out spectrophotometrically after acid digestion in a microwave.

PAR was measured using a LI-190 quantum sensor and a LI-250A light meter (Lincoln, Nebraska, U.S.A.), and illuminance was data-logged using a PCE-174 lux meter (Albacete, Spain). A Consort multi-logger (Belgium) equipped with a Consort DO probe (Belgium) and a Bioblock Scientific pH probe (France) were used for online measurement of the pH, temperature, and DO concentration of the HRAP cultivation broth and the outdoor batch reactors. The pH, temperature, and DO concentration in the batch reactors used to investigate TET sorption and the influence of pH were measured with a CyberScan pH 510 meter and a handheld OXI 330i oximeter (WTW, Germany).

3. Results and Discussion

3.1 Tetracycline removal in batch experiments

3.1.1 TET removal during full-day outdoor test

There was negligible TET removal in the MQ water control at night, which confirmed the absence of TET hydrolysis, TET sorption to the plastic reactor material or other abiotic TET degradation mechanisms (Figure 1). TET removal in the batch reactors containing active biomass or dead biomass was characterized by a rapid decrease in TET concentrations from 2.0 mg L^{-1} to 1.0-1.2 mg L^{-1} within an hour, followed by slow removal under dark conditions (Figure 1). Based on the initial rapid TET removal and the similar TET removals in the presence of active and the dead biomass during the night, TET sorption to biomass was hypothesized as the main TET removal mechanism. To test this hypothesis an independent test was conducted to investigate the recovery of sorbed TET from the biomass by extraction after incubation in the dark (Section 3.1.2).

Photodegradation was the dominant removal mechanism during the day (Figure 1), as TET removal rapidly increased upon sunlight exposure. Photodegradation was also reported as a dominant TET removal mechanism by de Godos et al. (2012), who studied TET fate in a lab-scale HRAP treating synthetic wastewater. Photodegradation can be further divided into direct or indirect photodegradation. Direct photodegradation occurs when the target pollutant degrades after absorbing light. In contrast, during indirect photodegradation the absorption of light by other dissolved organics generates reactive oxygen species, and these reactive species may subsequently degrade the target pollutant, contributing to the fate of emerging pollutants in surface waters (Beliakova et al., 2003; Challis et al., 2014; Niu et al., 2013; Wammer et al., 2011).

Tetracycline photodegradation in our experiment appeared to follow pseudo-first order kinetics, as is commonly reported (Beliakova et al., 2003; de Godos et al., 2012; Niu et al., 2013; Wammer et al., 2011), and our previous experiments supported the use of pseudo-first order kinetics as the most suitable for TET removal in HRAP systems (Norvill, 2016). Pseudo-first order kinetic rates for TET degradation under sunlight were based on points measured between 11 am to 3 pm, with average PAR of 1508 µmol m^{-2} s^{-1} (Figure 1). Thus, TET was degraded by direct photolysis in the MQ water control ($k = 2.8 \pm 0.3$ d^{-1}, $R^2 = 0.97$, $n = 3$) but the rate of TET photodegradation was 7 times greater in the presence of active biomass ($k = 19.2 \pm 5.9$ d^{-1}, $R^2 = 0.84$, $n = 3$), which indicated that indirect photodegradation (photo-oxidation) was involved. The TET photodegradation rate was slower in the presence of dead biomass ($k = 10.6 \pm 0.1$ d^{-1}, $R^2 = 1.00$, $n = 3$) than in the

298 presence of active biomass. Since the experiment in Section 4.1.2 demonstrated that
299 biodegradation was minimal, this difference was most likely due to the disruption of dead
300 biomass during autoclave treatment resulting in increased light attenuation compared to the
301 flocculated active biomass. Tests with filtered HRAP effluent (i.e. negligible biomass)
302 conducted under similar conditions (data not shown) determined that the HRAP biomass
303 exerted an insignificant effect on TET removal rates, which supported the conclusion that
304 biodegradation under light conditions was minimal compared to photodegradation (Norvill,
305 2016). Lower pH and DO concentrations, caused by the absence of photosynthetic activity,
306 might also have reduced TET removal rates in the presence of dead biomass (Figures S2
307 and S3, Supporting Information). pH and DO concentration effects on TET removal are
308 discussed further in Section 3.2.3. Temperatures during the batch test varied from 15°C at
309 8:00 to 40 °C at 15:00.
310
311 **3.1.2 TET sorption to biomass at different TET concentrations**
312 The extraction of HRAP biomass previously exposed to different TET concentrations (0.2
313 to 10 mg L^{-1}) confirmed the hypothesis that TET sorption onto the algal-bacterial biomass
314 was the major cause of the TET removal during darkness, with sorbed TET concentrations
315 from 0.2 to 4.2 mg g_{TSS}^{-1}. Mass balance calculations yielded total TET recoveries (sorbed
316 TET + aqueous TET) of 97 ± 14% (n = 5) and 97 ± 12% (n = 6), after 4 and 14 hours of
317 exposure to TET, respectively (Tables S1 and S2, Supporting Information). These high and
318 consistent TET recoveries confirmed that TET removal by biodegradation was negligible
319 relative to sorption at night (Section 3.1.1). Sorption at 14 h was best described by the
320 Freundlich isotherm (R^2 = 0.999); detailed discussion and data can be found in the

Supplementary Information Section S2.2 (Limousin et al., 2007). A preliminary sorption test with autoclaved biomass compared to non-autoclaved biomass was also conducted (data not shown). This preliminary test determined that the autoclaved biomass removed less TET than the non-autoclaved biomass, but this difference was due to the lower sorption capacity induced by autoclaving – the original TET was fully recovered by sorption extraction analysis in both tests (Norvill, 2016).

3.1.3 Influence of pH on TET removal

A batch experiment was conducted to evaluate the effect of pH on TET removal in the presence of active biomass under darkness. While TET removal was similar (Figure 2) in experiments conducted at pH 6 and unadjusted-pH (pH ~6.5-6.8), TET concentration immediately decreased at pH of 8.5 and 10.5. This rapid TET removal occurred within 30 s and was possibly due to hydrolysis, epimerization (other than 4epiTET) or sorption related mechanisms. As this rapid drop brought TET concentrations near the quantification limit, no subsequent observations could be made. A pH-mediated hydrolysis was hypothesized as the main mechanism responsible for TET fate at high pH values. However, further investigation is required to confirm the mechanisms affected by pH, which could be especially important for HRAPs that reach pH up to 11 during the day (Norvill et al. 2016). The average DO concentration and temperature recorded in the batch reactors with biomass were 0.9 ± 0.1 mg L^{-1} and 19.4 ± 1.0 °C (n = 16), respectively.

342 ***3.2 Continuous wastewater treatment in HRAP***

343 **3.2.1 HRAP performance**

344 COD and TOC removals ranging from 75 to 84% and 75 to 88%, respectively, were
345 recorded during all stages of operation (Table 2) while the removal of TN, PO_4^{3-} and TSS
346 ranged from 40 to 66%, 31 to 59%, and 63 to 89%, respectively (Table 2). This data
347 showed that HRAP operation was efficient to its primary WWT purpose, with a similar
348 performance to other HRAPs operated with domestic wastewater (Park et al., 2013;
349 Posadas et al., 2015a). High HRAP concentrations of NO_3^- and NO_2^- were associated with
350 low TN removals (Table 2), either due to increased nitrification competitively limiting NH_3
351 volatilization, or decreased denitrification allowing NO_3^- and NO_2^- accumulation (de Godos
352 et al., 2009; Ferrero et al., 2012; Sutherland et al., 2014). The removal of COD, TOC, and
353 inorganic carbon (IC) improved between Stage I and Stage II (Table 2), and these changes
354 were likely due to the stabilization of the algal and bacterial populations.

355

356 The decrease in HRT from 7 to 4 days (Stage II to Stage III) was associated with an
357 increase in biomass productivity from 4.5 ± 2.0 $g_{TSS}\,m^{-2}\,d^{-1}$ to 15.0 ± 1.7 $g_{TSS}\,m^{-2}\,d^{-1}$. The
358 lower productivity observed at 7 d HRT was likely due to IC limitation (algae consume
359 carbonates for growth) and higher biomass decay due to the increased cell residence time,
360 as the biomass concentration in the HRAP was similar at 7 and 4 d HRTs (Table 1). The
361 removals of COD, NH_4^+, TOC and TSS also decreased slightly from Stage II to Stage III as
362 HRT was decreased (Table 2). The decrease in TSS removal was attributed to the reduced
363 HRT in the clarifier and changes in biomass flocculation characteristics due to variations in
364 microalgae population structure. Changes in TET supply did not appear to cause

365 deterioration in HRAP performance, as most removal efficiencies increased or remained
366 similar between Stages I and II and between Stages III and IV. However, a decrease in
367 biomass settleability was recorded after the TET pulse tests were conducted (Section 3.2.3)
368 and TSS concentration in the clarifier thus decreased from ~15 g L^{-1} to ~ 5 g L^{-1} during
369 Stage IV, although the effluent TSS concentrations remain stable during Stages III and IV
370 (Table 2). de Godos et al. (2012) also reported biomass de-flocculation following TET
371 addition to a lab-scale HRAP, and this could impair biomass harvesting during full-scale
372 operation. Further analysis and data on HRAP performance, algal identification and
373 biomass composition are available in Section S3, Supporting Information, along with local
374 meteorological data.

375

376 **3.2.2 Tetracycline removal during continuous operation**

377 The experimentally measured TET influent concentration averaged 36 ± 2 µg L^{-1} (n = 8)
378 due to TET sorption to the suspended and colloidal solids present in wastewater. Since no
379 TET was detected in fresh wastewater samples, the TET removal efficiencies were
380 calculated based on theoretical influent concentration (aqueous + sorbed) of 100 µg TET L^{-1}.
381 TET was removed below the 2 µg L^{-1} quantification limit (>99%, Figure 3) during
382 process operation at 7 d HRT (Stage II). The average effluent TET concentrations increased
383 up to 3.4 ± 0.2 µg L^{-1} (95% CI) when the HRT was decreased to 4 d, which corresponded to
384 97 ± 1 % removal. In comparison, the overall TET removal typically observed in activated
385 sludge WWT varies broadly (e.g. 32-85% (Batt et al., 2007); 10-85% (Plosz et al., 2010);
386 24-100% (Michael et al., 2013)). Based on dominant photodegradation observed during the

387 batch test results (Section 3.1.1), TET concentrations in the HRAP were expected to
388 decrease during daylight. Indeed, there was a statistically significant ($p<0.01$) difference
389 between TET concentrations in the morning (4.2 ± 0.5 µg L^{-1}) and evening (2.5 ± 0.2 µg L^{-1})
390 when operated at 4 d HRT (Figure 3).
391
392 From the sorption extraction analysis the TET concentrations sorbed on the algal-bacterial
393 biomass were estimated to 4.0 $\mu g_{TET}\ g_{TSS}^{-1}$ at 7 d HRT and 12.5 $\mu g_{TET}\ g_{TSS}^{-1}$ at 4 d HRT..
394 Using the average biomass productivities recorded during Stages II and III (Table 1), TET
395 removal by sorption was calculated at 0.9% and 5.3% of the overall TET removal recorded
396 at 7 d HRT and 4 d HRT operation, respectively. This HRAP was operated with HRT equal
397 to SRT (sludge retention time). However, SRT can be increased via biomass recycling
398 (Park et al. 2013), which may have indirect effect on TET sorption, by altering the
399 productivity or the structure of the algal-bacterial population, the latter impacting the
400 biomass sorption properties.
401
402 A linear partition coefficient approximation (K_d = [sorbed]/ [aqueous TET]) is often used to
403 describe the sorption isotherm at equilibrium at low TET concentrations (Limousin et al.,
404 2007). Since aqueous TET concentrations were below the quantification limit during 7 d
405 HRT operation, a partition coefficient was only calculated during 4 d HRT operation. K_d
406 values in the range of 2.8-4.5 L g^{-1} were thus recorded during Stage III: these values were
407 higher than those achieved in the sorption batch tests performed during Stage IV (K_d = 0.5 -
408 2.2 L g^{-1}, Table S2, Supplementary Information), but comparable to previously reported
409 results in lab-scale HRAPs (K_d = 4.2 ± 0.2 L g^{-1}; de Godos et al., 2012). In comparison, K_d

values reported for TET sorption on activated sludge range from 0.47 to 8.4 L g^{-1} (Kim et al., 2005; Plósz et al., 2010; Prado et al., 2009). In view of the variabilities reported, the sorption characteristics of algal-bacterial biomass can be considered as similar to the sorption characteristics on the biomass generated during conventional biological treatment (e.g. activated sludge processes). No adverse effects on the algae activity (observed by daily pH and DO fluctuations) or the HRAP performance were noticed during continuous HRAP operation with TET added to the influent wastewater.

As WWT in HRAPs generates considerably more biomass than conventional biological WWT, HRAP WWT should also provide a larger sorption capacity for removing hydrophobic pollutants (Norvill et al., 2016). A high sorption capacity may enable the attenuation of night shock loadings and the removal of hydrophobic pollutants recalcitrant to photodegradation (experimental demonstration is required). However, where efficient pollutant photodegradation is taking place, the results suggest that overall sorption will be minimal (thus also minimizing sludge management hazards, as antibiotics can desorb from the sludge) (Kim et al., 2005; Norvill et al., 2016).

3.2.3 Tetracycline pulses in the pilot HRAP

The pulses of TET concentration were rapidly mitigated, showing that pilot HRAP operation was robust to fluctuations in TET influent load (Figure 4). TET was rapidly removed during sunlight exposure after the first TET pulse and TET removal slowed after 5:00 pm when the HRAP was no longer exposed to direct sunlight. The second TET pulse induced under dark conditions was also followed by a rapid disappearance of the aqueous

433 TET, but then TET concentrations stabilized around 30 µg L^{-1} overnight. TET removal
434 subsequently accelerated when the HRAP was exposed to direct sunlight the second day
435 (Figure 4). Based on the batch test results discussed in Section 3.1, the removal after the
436 first TET pulse was due to a combination of sorption and photodegradation but after the
437 second TET pulse sorption was the only applicable mechanism overnight, with TET
438 photodegradation beginning again the second day.
439
440 Based on the assumption that the HRAP was a well-mixed system, TET removal under
441 sunlight was best described by pseudo-first order kinetics (Table 3), measuring kinetic rates
442 between 11 am – 3 pm when the sunlight intensity was most consistent. Since sorption of
443 TET on the HRAP biomass had already approached equilibrium during the night, the rates
444 of TET removal recorded under sunlight during the second day were consistently lower
445 than the rates of TET removal recorded during the first day, when rapid TET removal by
446 both sorption and photodegradation occurred (Table 3). As photodegradation decreased
447 aqueous TET concentrations during sunlight exposure, desorption of TET from the biomass
448 likely occurred. Therefore, TET desorption likely explains the difference in the daytime
449 TET removal rates recorded during the first and second days of each pulse test.
450
451 A mathematical model developed to predict TET removal based on sorption and
452 photodegradation also demonstrated that photodegradation was directly proportional to the
453 recorded PAR (i.e. the pseudo-first order rates reported here were dependent on sunlight
454 intensity) (Norvill, 2016). The mathematical model was not included in this article due to
455 space limitations.

The comparison of TET removal rates against environmental variables (light, pH, DO, temperature) revealed that the highest TET removal rates were associated with high DO concentrations (>6.9 mg-O_2 L^{-1}), high pH (>7.1) and high PAR (>1343 μmol m^{-2} s^{-1}) (Figure S5, Supporting Information). While these 3 parameters are interdependent (high light irradiance generally causes both pH and DO to increase due to photosynthesis), a possible causality between DO concentrations and TET removal may be related to the generation of reactive oxygen species in indirect photodegradation mechanisms (Sandvik et al., 2000; Vaughan and Blough, 1998). Alternatively, a high pH may enhance TET removal via change in the ionic state of TET (which has a pKa ~7.8; Qiang and Adams, 2004). Direct photodegradation of TET has indeed been reported to increase at high pH, although no research was found that investigated whether indirect TET photodegradation rates also increase at high pH (Chen et al., 2008; López-Peñalver et al., 2010; Niu et al., 2013; Wammer et al., 2011). The decreased biomass settleability after the initial pulse tests (Section 3.2.1) may also have affected TET removal in the last three pulse experiments (Table 3), via differences in light attenuation (algal floc disruption) and/or sorption characteristics. The effect of pH and DO concentration upon the mechanisms of TET removal in HRAP therefore requires further study.

4. Conclusions

Overall, TET was effectively removed during pilot HRAP operation at 7 d and 4 d HRT under summer conditions, although the reliance on photodegradation may result in reduced

478 removal efficiencies during winter. These results provide the first demonstration in the
479 literature of efficient tetracycline removal during relevant HRAP operation outdoors (low
480 pollutant concentration and real wastewater) and indicate that algal based WWT provides
481 higher removal capacity (via indirect photodegradation and sorption) than conventional
482 biological WWT, although the removal of other antibiotics and emerging pollutants in
483 HRAPs must be assessed in further research.
484

485 **5. Description of Supporting Information**

486 The supporting information includes an image and schematic of the pilot HRAP operation,
487 the environmental conditions for the batch tests, mass balance calculations for the sorption
488 test, a comparison of sorption isotherm models used to describe the sorption near
489 equilibrium, tables of the monitored HRAP variables and the comparison of TET removal
490 rates with monitored environmental variables during the TET pulse tests.
491

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498

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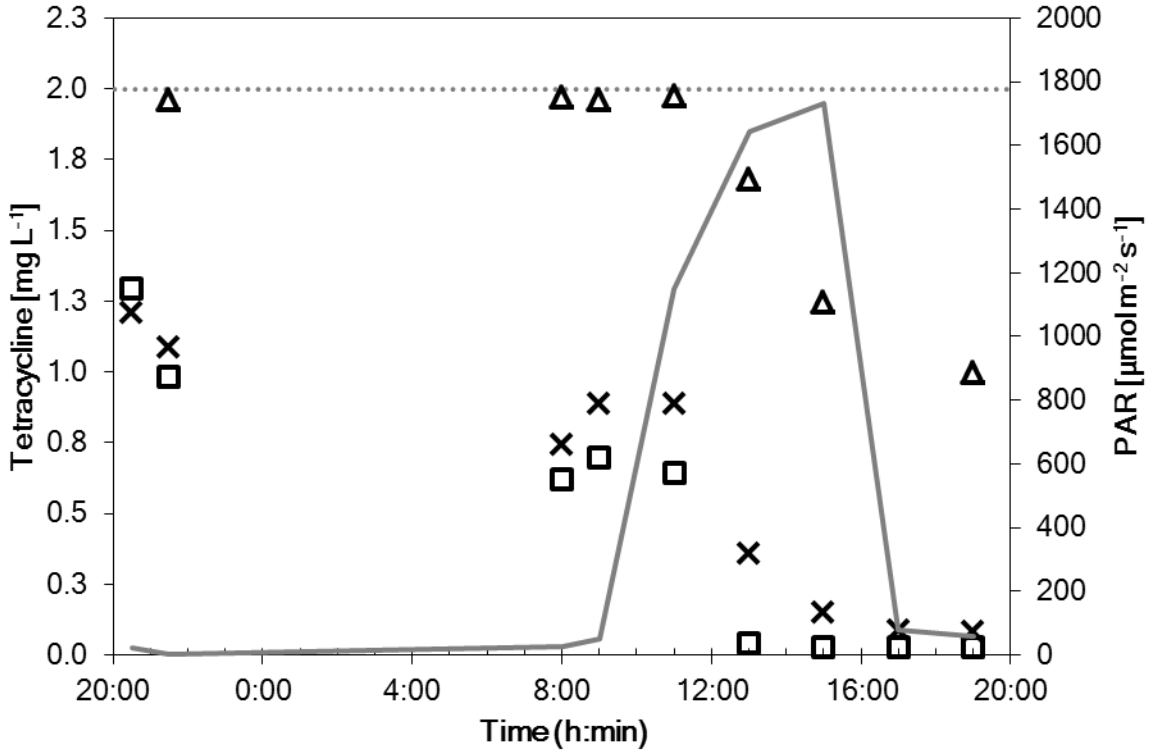


666

667 **Figure 1.** Tetracycline concentration and PAR in outdoor batch experiments conducted
668 with biomass sourced from the pilot HRAP during 4 d HRT operation: MQ water control (
669 Δ), dead biomass (×), active biomass (□). The dotted horizontal line shows the
670 theoretical initial TET concentration. Surface solar irradiance measured during the
671 experiment is shown on the secondary axis (continuous grey line).

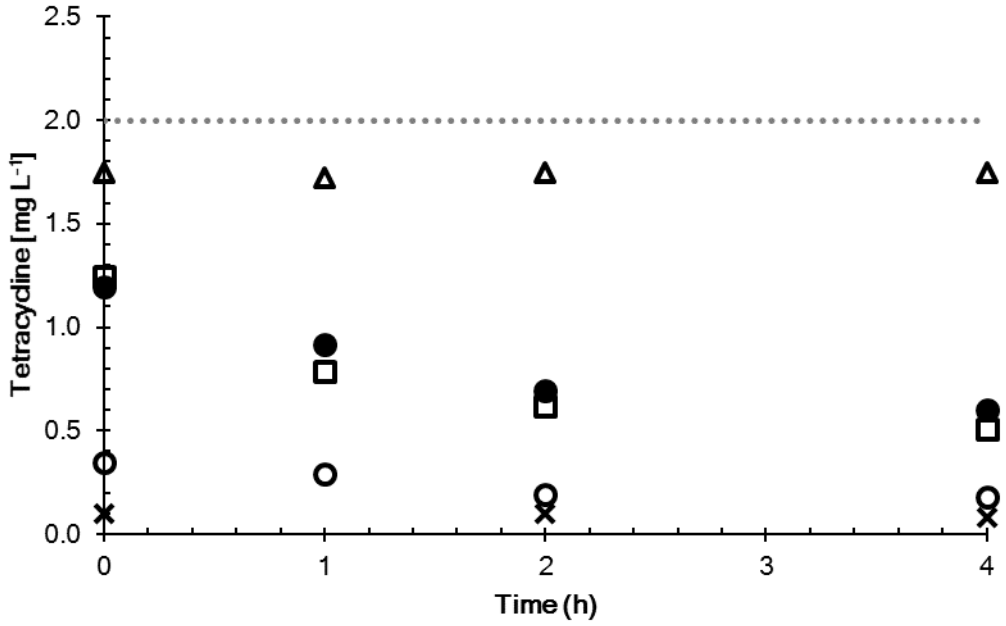
672

673 **Table 1**

674 Environmental parameters and productivity recorded during HRAP operation.[a]

	Units	Stage I	Stage II	Stage III	Stage IV
HRAP conditions		Start-up (no TET)	Continuous TET	Continuous TET	Continuous or Pulsed TET
Pseudo-steady-states[b]		26th May - 16th June (21 days)	17th June - 21st July (34 days)	29th July - 6th Aug (8 days)	7th Aug - 3rd Sep (27 days)
HRT	days	7	7	4	4
Average Evaporation	L m^{-2} d^{-1}	2.9 ± 4.6 (21)	7.7 ± 2.2 (32)	10.4 ± 7.8 (10)	6.7 ± 2.3 (27)
Average clear-sky GHI[c]	W h m^{-2}	8469	8583	8160	7236
Low[d] Temperature	°C	15.0 ± 2.2 (17)	16.5 ± 0.7 (33)	15.6 ± 1.2 (8)	15.3 ± 0.7 (28)
High[d] Temperature	°C	25.9 ± 3.3 (17)	32.8 ± 1.0 (33)	32.4 ± 1.1 (8)	29.8 ± 1.6 (28)
TSS	g L^{-1}	1.1 ± 0.3 (6)	1.1 ± 0.1 (10)	1.3 ± 0.6 (3)	1.2 ± 0.1 (8)
Low[d] pH	[ ]	5.5 ± 0.3 (17)	5.6 ± 0.1 (32)	5.9 ± 0.3 (8)	6.2 ± 0.1 (25)
High[d] pH	[ ]	6.5 ± 0.2 (17)	6.9 ± 0.2 (32)	7.5 ± 0.5 (8)	7.3 ± 0.2 (25)
Low[d] O_2	mg L^{-1}	2.2 ± 1.5 (14)	0.6 ± 0.1 (33)	0.3 ± 0.1 (8)	0.2 ± 0.1 (28)
High[d] O_2	mg L^{-1}	12.6 ± 0.8 (14)	9.8 ± 0.9 (33)	10.0 ± 2.8 (8)	8.0 ± 0.8 (28)
Productivity	g m^{-2} d^{-1}	7.4 ± 5.3 (6)	4.5 ± 2.0 (10)	15.0 ± 1.7 (3)	15.9 ± 4.6 (8)

675 [a] Data is shown as the mean value ± 95% CI (n). [b] The days listed in brackets show the
676 duration of the pseudo-steady-state period only – the total time under each set of operating
677 conditions is listed in Section 2.3.1. [c] Global solar irradiance to a horizontal plane, CAMS
678 radiation service www.soda-pro.com; accessed May 2016. [d] 'Low' and 'High' parameters
679 refer to the 5th and 95th percentiles of 24-hour datalogged values. Temperatures given are
680 from the data-logged water temperatures in the HRAP. These percentiles are reported rather
681 than max/min in order to remove potential outliers.



682

683 **Figure 2.** Time course of tetracycline concentrations during the incubation of algal-

684 bacterial biomass under darkness and under controlled pH conditions at pH 6 (●), pH 8.5

685 (○) and pH 10.5 (×) or under uncontrolled pH (pH 6.5-6.8) (□) and in MQ water (Δ).

686 The horizontal dotted line shows the theoretical initial TET concentration.

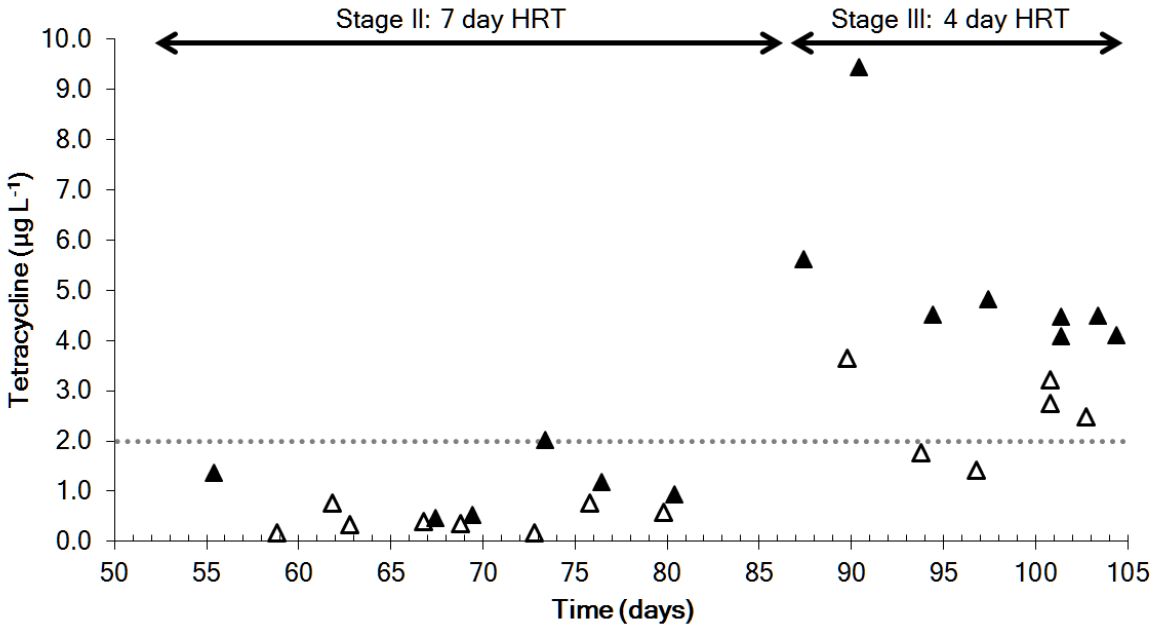
687

Table 2

HRAP performance during pseudo-steady state conditions in each operational stage. Data is shown as the mean value ± 95% CI (n).

Stage	I			II			III			IV		
Characteristic	Influent [mg L^{-1}]	Effluent [mg L^{-1}]	RE (%)	Influent [mg L^{-1}]	Effluent [mg L^{-1}]	RE (%)	Influent [mg L^{-1}]	Effluent [mg L^{-1}]	RE (%)	Influent [mg L^{-1}]	Effluent [mg L^{-1}]	RE (%)
COD	787 ± 392 (6)	160 ± 26 (6)	75 ± 26 (5)	621 ± 63 (8)	183 ± 18 (9)	84 ± 2 (9)	669 ± 258 (3)	479 ± 241 (3)	78 ± 10 (3)	690 ± 104 (8)	407 ± 31 (8)	79 ± 5 (8)
$N\text{-}NH_4^+$	56 ± 16 (6)	5 ± 2 (6)	95 ± 2 (5)	66 ± 13 (10)	14 ± 4.2 (10)	89 ± 2 (10)	72 ± 1 (3)	14 ± 5 (3)	85 ± 4 (3)	59 ± 15 (8)	12 ± 3 (8)	84 ± 3 (8)
$N\text{-}NO_2^-$	not detected	42 ± 7 (6)	n.a.	not detected	39 ± 12 (10)	n.a.	not detected	24 ± 10 (3)	n.a.	not detected	6 ± 6 (7)	n.a.
$N\text{-}NO_3^-$	not detected	3 ± 4 (6)	n.a.	not detected	30 ± 18 (10)	n.a.	not detected	16 ± 4 (3)	n.a.	not detected	34 ± 14 (8)	n.a.
$P\text{-}PO_4^{-3}$	10 ± 10 (6)	7.5 ± 9 (5)	51 ± 8 (4)	10 ± 7 (10)	8.9 ± 8 (7)	49 ± 6 (7)	10 ± 25 (3)	6 ± 53 (2)	59 ± 7 (2)	8 ± 8 (7)	7 ± 7 (7)	31 ± 11 (7)
TN	117 ± 10 (5)	58 ± 6 (6)	68 ± 3 (5)	102 ± 9 (9)	91 ± 16 (9)	47 ± 15 (9)	70 ± 20 (3)	54 ± 16 (3)	39 ± 24 (3)	93 ± 11 (8)	59 ± 11 (8)	46 ± 20 (8)
TOC	176 ± 44 (6)	32 ± 3 (6)	81 ± 19 (6)	165 ± 11 (9)	42 ± 5 (9)	88 ± 4 (9)	147 ± 113 (3)	44 ± 5 (3)	75 ± 20 (3)	171 ± 19 (8)	46 ± 11 (8)	77 ± 5 (8)
IC	87 ± 19 (6)	12 ± 7 (6)	90 ± 5 (6)	87 ± 17 (9)	6 ± 1 (9)	95 ± 1 (9)	82 ± 7 (3)	17 ± 8 (3)	84 ± 8 (3)	88 ± 9 (8)	21 ± 8 (8)	81 ± 7 (8)
TSS[a]	119 ± 27 (6)	16 ± 4 (6)	89 ± 5 (6)	130 ± 16 (10)	22 ± 6 (10)	90 ± 3 (10)	113 ± 47 (3)	51 ± 22 (3)	63 ± 33 (3)	122 ± 42 (8)	57 ± 16 (8)	64 ± 16 (7)

[a] Removal efficiency for TSS was calculated based on influent and clarified effluent concentrations and their respective flow-rates to account for evaporation. It thus includes but is not limited to the settling efficiency of the clarifier.



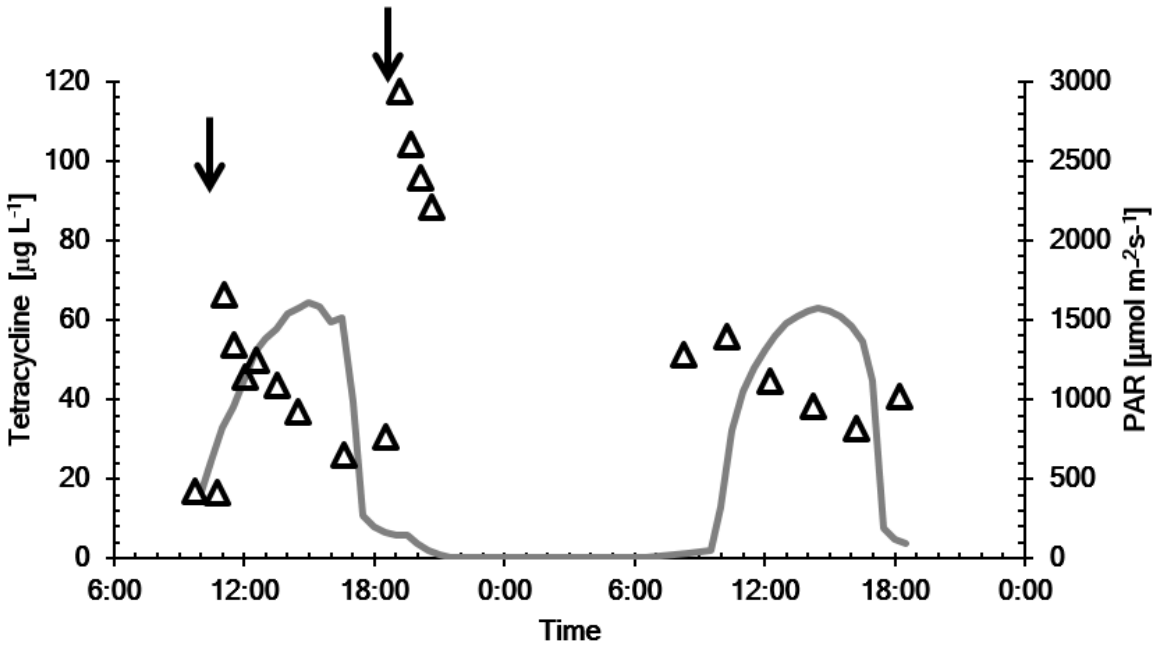
693

694 **Figure 3.** Time course of tetracycline concentrations in the 180L HRAP in the morning

695 (▲) and evening (Δ) during continuous supplementation of tetracycline to the wastewater

696 influent at 100 µg TET L^{-1}. The quantification limit is represented by a dotted line.

697



698

699 **Figure 4.** Time course of the PAR (continuous line, half-hour averages) and TET

700 concentration (Δ) during two consecutive TET pulses supplied to the HRAP (27th Aug

701 2015). Vertical arrows represent TET supplementation to the HRAP cultivation broth.

702

Table 3

Summary of pseudo-first order TET kinetic constants (k_1) describing TET removal under solar irradiation (10 am - 4 pm) during the pulse tests

	Day 1 $k_1 \pm$ st.error [d^{-1}] (R^2, n)	**Day 2** $k_1 \pm$ st.error [d^{-1}] (R^2, n)	**Day 1** Average PAR (peak PAR) [µmol m^{-2} s^{-1}]	**Day 2** Average PAR (peak PAR) [µmol m^{-2} s^{-1}]
7-Aug	5.6 ± 1.9 (0.70, 6)	1.8 ± 0.3 (0.93, 3)	1329 (1839)	1114 (1851)
13-Aug	9.9 ± 1.8 (0.82, 9)	6.2 ± 0.2 (0.99, 3)	946 (2508)	754 (1931)
18-Aug	7.4 ± 1.8 (0.72, 8)	no evening pulse	1285 (1655)	n.a.
20-Aug	3.2 ± 0.4 (0.90, 9)	1.4 ± 0.3 (0.79, 4)	1330 1614)	1229 (1786)
27-Aug	2.4 ± 0.4 (0.83, 8)	0.9 ± 0.4 (0.56, 5)	1288 (1766)	1184 (1593)
31-Aug	no morning pulse	0.3 ± 0.1 (0.78, 6)	n.a.	1026 (1745)