Aerobic Toluene Degraders in the Rhizosphere of a Constructed Wetland Model Show Diurnal Polyhydroxyalkanoate Metabolism

Vanessa Lünsmann,^{a,b} Uwe Kappelmeyer,^b Anja Taubert,^b Ivonne Nijenhuis,^c Martin von Bergen,^{a,d,e} Hermann J. Heipieper,^b Jochen A. Müller,^b Nico Jehmlich^a

Helmholtz Centre for Environmental Research-UFZ, Department of Molecular Systems Biology, Leipzig, Germany^a; Helmholtz Centre for Environmental Research-UFZ, Department of Environmental Biotechnology, Leipzig, Germany^b; Helmholtz Centre for Environmental Research-UFZ, Department of Isotope Biogeochemistry, Leipzig, Germany^c; University of Leipzig, Faculty of Biosciences, Pharmacy and Psychology, Institute of Biochemistry, Leipzig, Germany^d; Department of Biotechnology, Chemistry and Environmental Engineering, University of Aalborg, Aalborg, Denmark^e

ABSTRACT

Constructed wetlands (CWs) are successfully applied for the treatment of waters contaminated with aromatic compounds. In these systems, plants provide oxygen and root exudates to the rhizosphere and thereby stimulate microbial degradation processes. Root exudation of oxygen and organic compounds depends on photosynthetic activity and thus may show day-night fluctuations. While diurnal changes in CW effluent composition have been observed, information on respective fluctuations of bacterial activity are scarce. We investigated microbial processes in a CW model system treating toluene-contaminated water which showed diurnal oscillations of oxygen concentrations using metaproteomics. Quantitative real-time PCR was applied to assess diurnal expression patterns of genes involved in aerobic and anaerobic toluene degradation. We observed stable aerobic toluene turnover by *Burkholderiales* during the day and night. Polyhydroxyalkanoate synthesis was upregulated in these bacteria during the day, suggesting that they additionally feed on organic root exudates while reutilizing the stored carbon compounds during the night via the glyoxylate cycle. Although mRNA copies encoding the anaerobic enzyme benzylsuccinate synthase (*bssA*) were relatively abundant and increased slightly at night, the corresponding protein could not be detected in the CW model system. Our study provides insights into diurnal patterns of microbial processes occurring in the rhizosphere of an aquatic ecosystem.

IMPORTANCE

Constructed wetlands are a well-established and cost-efficient option for the bioremediation of contaminated waters. While it is commonly accepted knowledge that the function of CWs is determined by the interplay of plants and microorganisms, the detailed molecular processes are considered a black box. Here, we used a well-characterized CW model system treating toluene-contaminated water to investigate the microbial processes influenced by diurnal plant root exudation. Our results indicated stable aerobic toluene degradation by members of the *Burkholderiales* during the day and night. Polyhydroxyalkanoate synthesis in these bacteria was higher during the day, suggesting that they additionally fed on organic root exudates and reutilized the stored carbon compounds during the night. Our study illuminates microbial processes occurring in the rhizosphere of an aquatic ecosystem.

Constructed wetlands (CWs) are well-established treatment systems for the bioremediation of contaminated waters (1), including industrial effluents originating from petroleum refineries and their associated contaminated sites (2, 3). Aromatic hydrocarbons present in those wastewaters can effectively be removed in CWs (4–7), largely driven by the interplay of plant physiology and microbial degradation processes in the rhizosphere (8, 9). As in any planted habitat, the rhizosphere of CWs is a complex environment with increased abundance and activity of microorganisms compared to those in the surrounding zones (10). It is shaped by the roots and plant-derived exudates, such as sugars, amino acids, organic acids, and phenolic compounds (11, 12). In addition, the helophytes usually grown in CWs channel oxygen into their roots and the rhizosphere through a specific tissue, the aerenchyma (13), thereby fostering aerobic microbial processes. The release of oxygen and organic carbon by any plant root depends on photosynthesis and therefore may exhibit diurnal oscillations (14, 15). These oscillations were indeed described for root exudation by *Juncus effusus*, a common wetland plant (16, 17). Fluctuating redox conditions in the rhizosphere of CWs, cooccurring with the day-night regimen (18), as well as the influence of these diurnal

fluctuations on various redox transformation processes involving nitrogen compounds (19) and iron (20) have been reported. Yet, while several studies described diurnal patterns of abiotic parameters in CW systems, studies on diurnal patterns of microbial activity in CW systems are scarce (21).

Planted fixed-bed reactors (PFRs) are CW model systems in which diurnal changes of abiotic parameters have frequently been

Received 17 February 2016 Accepted 26 April 2016

Accepted manuscript posted online 29 April 2016

Citation Lünsmann V, Kappelmeyer U, Taubert A, Nijenhuis I, von Bergen M, Heipieper HJ, Müller JA, Jehmlich N. 2016. Aerobic toluene degraders in the rhizosphere of a constructed wetland model show diurnal polyhydroxyalkanoate metabolism. *Appl Environ Microbiol* 82:4126–4132. doi:10.1128/AEM.00493-16.

Editor: J. E. Kostka, Georgia Institute of Technology

Address correspondence to Hermann J. Heipieper, hermann.heipieper@ufz.de.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.00493-16>.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

observed (22, 23). Such PFR systems were further used to investigate microbial degradation of the aromatic hydrocarbon toluene by isotope fractionation and DNA microarray analysis, 16S rRNA amplicon sequencing (24), and protein-based stable isotope probing (protein-SIP) (25). These studies revealed that toluene was predominantly or even exclusively degraded aerobically by members of the *Burkholderiales* via a sequential monooxygenation of the aromatic ring followed by ring cleavage through an extradiol dioxygenase. Aerobic microbes that catabolize toluene via this pathway apparently have a competitive advantage at low-oxygen partial pressure over bacteria that employ the TOL pathway or the dioxygenase pathway or carry out toluene degradation via protocatechuate (26, 27).

The protein-SIP study further showed that about 70% of the carbon in newly synthesized *Burkholderiales* proteins was derived from toluene (25). Some of those labeled proteins were involved in metabolism of the common carbon storage polymer polyhydroxyalkanoate (PHA), but the experimental setup did not allow detailed conclusions on the dynamics of toluene degradation and carbon storage. Additionally, it was found that the microbial community in the PFRs apparently also possessed the potential for anaerobic toluene degradation via benzylsuccinate and benzoyl-coenzyme A (CoA) (28), as respective key genes were rather abundant. However, no evidence for *in situ* anaerobic toluene degradation was found by proteomics or isotope fractionation.

The main objective of this study was to query whether diurnal oscillations of plant-derived compounds affect microbial aerobic and potentially also anaerobic toluene degradation in the PFR rhizosphere and whether this would also impact the subsequent metabolic routes, including PHA metabolism. In addition to recording day-night profiles of abiotic PFR conditions, we analyzed the mRNA abundances of genes associated with the toluene monooxygenase pathway as well as the gene for benzylsuccinate synthase (*bssA*) as a proxy for anaerobic toluene degradation via quantitative real-time PCR (qPCR). Furthermore, diurnal changes of protein abundances were comparatively analyzed by metaproteomics. Such investigations of microbial processes will help to further unravel the “black box” of CWs and will aid the design of wetland systems.

MATERIALS AND METHODS

Continuous-flow PFR. The PFR is a constructed wetland model system that has been running with toluene as the sole external carbon source for 5 years (24) (see also Fig. S1 in the supplemental material). The PFR is composed of a metal basket (diameter, 26 cm; height, 28 cm) filled with approximately 20 kg of gravel (particle size, 2 to 4 mm), which is inside a cylindrical glass vessel (30 by 30 cm). The CW model was designed analogously to a continuously stirred tank reactor in order to maintain macrogradient-free conditions in the rhizosphere. The reactor was planted with soft rush, *Juncus effusus*, and was closed on top by a Teflon lid with five openings for the plants and the sampling cannulas (Glasgerätebau Ochs; length, 20 cm; diameter, 2 mm). A continuous inflow of distilled water plus trace elements and toluene (50 mg/liter) was supplied by a plunger pump (Reglo-CPF; Ismatec). Inorganic nutrients (134 mg/liter nitrate, 19 mg/liter phosphate, 26 mg/liter ammonium) were added to the inflow by a syringe pump (KDS 200; DK Scientific, Inc., USA). The rhizospheric pore water volume was approximately 10 liters, and a hydraulic retention time of 5 days was maintained by an internal circulation flow (MCP; Ismatec) coupled to a water-level control system. From the circulation flow, redox potential (2ME-2G-PtK-1; Jumo), dissolved oxygen concentration (SIPAN 34 and 7MA3100-8EF; Siemens), and pH (2GE-2-G-U-1; Jumo) were monitored continuously. Evapotranspiration was

used as indicator for plant health and thus as a qualitative parameter of photosynthesis. The amount of water that was evapotranspired by the plants was calculated twice per week by determining inflow and outflow volumes.

Sampling from the CW model system. In order to assess if diurnal patterns of microbial processes occur in the PFR, samples were taken at times when the conditions in the rhizosphere differed the most. Day samples were taken at 10 a.m. during a peak of oxygen concentration, and the night samples were taken at midnight, when the oxygen concentration reached a low point. Sampling was repeated on three consecutive days and nights in July 2014 to cover natural day-to-day variations. At each time point, samples for RNA and protein extraction and determinations of toluene, dissolved organic carbon (DOC), inorganic carbon, ammonium, and nitrate were taken. To cover the spatial variability in the CW model, pore water was sampled at five different positions from the middle of the PFR using sampling cannulas. To detach biofilms from gravel and roots, the water was flushed up and down through the cannulas prior to the sampling. The five samples were pooled and were considered one replicate of the CW model. For RNA extraction 30 ml of pore water was taken (in total, 150 ml) from each cannula and pooled onto one filter (0.2 μ m, Supor200; Pall Life Sciences). For protein extraction, 5 ml of pore water was taken from each cannula and pooled onto one filter. The flowthrough was used for analysis of DOC, inorganic carbon, ammonium, and nitrate concentrations. For toluene analysis, 5 ml of pore water was taken separately from the CW model.

RNA extraction and cDNA synthesis. The filters were placed in a 50-ml Falcon tube, immediately frozen with liquid nitrogen, and stored at -80°C until extraction.

Total RNA was extracted from the filters using the RNeasy minikit (Qiagen). Biomass was detached from the filters by horizontal vortexing for 2 min after addition of 980 μ l RLT buffer (from the RNeasy minikit), 20 μ l β -mercaptoethanol, and two spatulas of glass beads (particle size, <160 μ m; G4649-10G; Sigma). The suspension was transferred to MP Bio lysing matrix tubes (FastPrepLysing matrix B; MP Biomedicals), and cells were disrupted for 40 s at 6 m/s on a FastPrep-24 instrument (MP Biomedicals). Supernatants were transferred to a new tube, and an equal volume of 70% ethanol was added. Subsequently, the manufacturer's protocol for RNA extraction from animal tissues was followed. RNA was eluted in two steps in a final volume of 70 μ l of RNase-free water. DNA contaminations were removed by double treatment with DNase (DNA-free DNA removal kit; Ambion, Life Technologies). RNA concentrations were determined spectrophotometrically with a NanoDrop (ND-1000; Peqlab).

Per sample, two batches of total RNA with 200 ng each were transcribed into cDNA using the Omniscript reverse transcriptase kit (Qiagen) with random hexamer primers (for 16S rRNA quantification) and with a mix of reverse primers targeting the genes of aerobic and anaerobic toluene degradation (see Table S1 in the supplemental material).

Quantitative real-time PCR. To quantify the expression of catabolic genes involved in the monooxygenase pathway of toluene degradation (*tmo*, *phe*, and the C23O-encoding gene) and anaerobic toluene degradation (*bssA*), cDNA samples from the CW model were analyzed with the respective qPCR assay (see Table S1 in the supplemental material). For intrasample normalization, 16S rRNA abundance was quantified. Absolute quantification assays were run in 96-well plates using an Applied Biosystems cyclor (StepOnePlus real-time PCR system) and the Kapa SYBR Fast qPCR master mix. Each cDNA sample was measured in triplicate; a negative control of the reverse transcription was analyzed to ensure the absence of genomic DNA. Dilution series of every target gene were measured as the quantification standard, as described previously (25).

Protein extraction. The protein extraction was done essentially as described previously (25), but horizontal vortexing was replaced by treatment in a FastPrep-24 instrument (MP Biomedicals) for 40 s at 6 m/s. Tryptic in-gel digestion was performed, and peptides were purified with SOLA μ SPE plates (Thermo Scientific). The peptide concentration was

determined with a NanoDrop (ND-1000; Peqlab), and, per sample, 1.3 µg of peptide lysate was dissolved in 15 µl of 0.1% formic acid and measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

LC-MS/MS measurements and protein identification. Mass spectrometry was performed on an Orbitrap Fusion MS (Thermo Fisher Scientific, Waltham, MA, USA) (described in detail in the supplemental material). Briefly, 5 µl of peptide lysates was separated on an ultrahigh-performance liquid chromatography system (Ultimate 3000; Dionex/Thermo Fisher Scientific, Idstein, Germany) equipped with a reverse-phase C₁₈ column coupled to a TriVersa NanoMate source (Advion, Ltd., Harlow, United Kingdom) in LC chip coupling mode. The acquired MS spectra were processed using the SequestHT algorithm and precursor ion detector (for label-free quantification) in Proteome Discoverer (v.1.4.0288; Thermo Scientific). Protein intensities were log₂ transformed and divided by the median log₂ protein intensity of each sample. To assess further the functions of the different bacterial community members, we assigned the identified proteins to clusters of orthologous groups (COGs). Proteins involved in toluene degradation are not categorized into COGs and thus were assigned manually (29). The lowest common phylogenetic ancestor of each protein group was classified using the Prophan pipeline (<http://www.prophan.de/>).

Toluene, DOC, inorganic carbon, ammonium, and nitrate analyses.

For determination of DOC, inorganic carbon, nitrate, and ammonium in the pore water of the CW model, the 0.2-µm flowthrough of RNA sampling was used. DOC and inorganic carbon were analyzed using a TOC analyzer (multi N/C 2100; Analytik Jena). Ammonium and nitrate concentrations were determined by ion chromatography (Dionex ICS-5000; columns, CS12, 2 by 250 mm for ammonium, and AS18, 2 by 250 mm for nitrate) using a UV detector at a wavelength of 215 nm for nitrate and a conductivity detector for ammonium. The self-regenerating suppressors ASRS-Ultra 2 mm (nitrate) and CBES-I 2 mm (ammonium) were applied. Toluene concentration was measured by headspace gas chromatography (GC)-flame ionization detection (FID), as described elsewhere (24).

RESULTS AND DISCUSSION

Stable conditions and diurnal fluctuations in the rhizosphere of the CW model. The CW model is a natural microbial ecosystem transferred to the laboratory. It has been running with toluene as the sole external carbon and energy for several years and been studied comprehensively in the past (24, 25). By protein-SIP, members of the *Burkholderiales* were identified as key players of toluene degradation. The findings of these previous studies were used as the knowledge base for the results described below. Due to its complexity, we focused on the in-depth analysis of one model wetland rather than investigating several systems.

The parameters of the experiment as well as the sampling time points are depicted in Fig. 1. During the experiment, evapotranspiration by the plants was about 20 g water/h, which is in the range of healthy, photosynthetically active *J. effusus* in this CW model system (data not shown). Oxygen concentrations in the rhizosphere showed diurnal variations, with peaks of oxygen at 10 a.m. (around 0.4 mg/liter) and low points at midnight (0.1 mg/liter). DOC concentrations in the pore water were essentially constant around 3.8 ± 0.7 mg/liter and revealed that other carbon sources, such as root exudates and derivatives thereof, were available to the rhizospheric microbial community (11, 16). Although we did not assess the composition of the root exudates in day and night samples, it can be assumed that these showed diurnal patterns comparable to the fluctuating oxygen concentrations (17).

To ensure that sufficient toluene was available to all potential degraders, the model wetland was operated at high toluene inflow concentrations of about 50 mg/liter. At this operational regimen, toluene was detected in the outflow at concentrations ranging

FIG 1 Parameters for the PFR during the 3 days of the diurnal experiment. Redox potential and oxygen concentration were monitored, and sampling points for day (●) and night (▼) are indicated. The solar radiation (upper part of the graph) defines the day and night rhythm.

between 0.5 and 2 mg/liter, without any diurnal dynamics. The redox potential was constant around 450 mV, which is favorable for aerobic processes (30). Low nitrate concentrations in the outflow (4.5 ± 1.2 mg/liter corresponding to about 3% of the inflow concentration) could be attributed to denitrification, which is the dominant nitrate removal process in wetlands (31). The microbial community in the rhizosphere of this wetland was previously analyzed and contained many potential denitrifying genera (32), such as *Burkholderia*, *Ralstonia*, *Magnetospirillum*, and *Rhizobium* (25). Ammonia removal (outflow, 12.8 ± 2.2 mg/liter, about 49% of the inflow concentration) could be explained by assimilation through plant and microorganisms (33) as well as by nitrification processes (34). Phosphate concentrations were always below the limit of detection of 1 mg/liter (see Table S2 in the supplemental material).

Rhizospheric microbial community exhibits stable protein profiles during the day and night. In the day ($n = 3$) and night ($n = 2$) samples, we identified in total 1,193 protein groups, showing a high overlap, with a mean of 779 ± 86 identifications per sample (see Data Set S1 in the supplemental material). A total of 553 proteins were identified in at least two replicates of the day and night samples and were used for further quantitative comparison. Fold changes (FC) and P values were calculated. Only 32 proteins (3% of all identified proteins) showed significant fold changes, with P values of <0.05 (see Table S3 in the supplemental material). In comparison to other metaproteomic studies, where 10 to 15% of all identified proteins were significantly regulated (35, 36), the number of regulated proteins in this study is distinctly small and implies a stable metaproteome profile of the rhizospheric community. Remarkably, more than 20% of these significantly regulated proteins were involved in PHA metabolism or the glyoxylate cycle and were also previously identified (25) (Fig. 2 and 3).

Metaproteomics revealed a protein profile similar to the one

FIG 2 Log₂ fold change of selected proteins identified in the model wetland. Proteins belonging to *Burkholderiales*, *Rhizobiales*, and *Corynebacteriales* are colored as indicated. Significant differences between protein groups (testing the group fold changes against all other fold changes) are indicated by asterisks (top of the figure). The number of proteins of each boxplot is given at the bottom of the figure.

FIG 3 Dynamics of different protein abundances during the diurnal cycles. Protein abundances were normalized to those in the first-day sample (▼).

observed in our earlier study, indicating the stability of the system (25). About 40% of the phylogenetically assigned proteins belonged to the *Burkholderiales*; more than 20% of the catabolic enzymes of this order were involved in aerobic toluene degradation via toluene monooxygenase, phenol hydroxylase, *meta* cleavage by catechol 2,3-dioxygenase, and further metabolism to acetyl-CoA by 2-hydroxymuconic semialdehyde dehydrogenase and acetaldehyde dehydrogenase. Figure 2 shows the combined fold changes of proteins with the same function but different taxonomic origins. For most relevant processes, such as toluene degradation, denitrification, transport, and central carbon metabolism, no significant differences in protein abundance were found between day and night. Proteins involved in denitrification, such as nitrate, nitrite, and nitrous oxide reductases, showed constant protein levels during day and night, and some were affiliated with *Rhodospirillales* and *Burkholderiales* (3 and 5 of 18 proteins, respectively), while for the other proteins, no detailed phylogenetic assignment was possible. Most enzymes of the citric acid cycle as well as ABC transporters (median log₂ FC, -0.143) showed stable abundances as well. Different trends were observed for transport proteins, where porins were grouped into OmpA- and OmpC-like porins (see Fig. S2 in the supplemental material). OmpC-like porins showed no diurnal differences (median log₂ FC = -0.096).

FIG 4 Mean (of 3 days/nights) abundances of mRNA copies of toluene monooxygenase, phenol hydroxylase, catechol 2,3-dioxygenase, and benzylsuccinate synthase. All mRNA abundances were normalized by the amount of rRNA copies quantified in each sample.

These porins have a smaller inner diameter than OmpA (see below) and other porins (37) and protect the bacteria from environmental stress, such as acidic conditions (38) or solvent stress (39). The supplied toluene as well as the acidic pH (5.6) in the CW model can explain the constant levels of many OmpC-like porins. The majority of transport proteins were matched to *Burkholderiales* (23 proteins) or *Rhizobiales* (15 proteins).

Stable abundances of genes and proteins involved in aerobic toluene degradation during day and night. Toluene degradation was stable during the day and night despite fluctuating oxygen concentrations. Transcript levels of toluene degradation genes involved in the dominant ring-hydroxylating pathway (24, 25), namely, toluene monooxygenase, (*tmoA*), phenol hydroxylase (*phe*), and catechol 2,3-dioxygenase (C23O-encoding gene) were determined by quantitative real-time PCR. Transcript abundances were normalized for each sample to the corresponding copy numbers of 16S rRNA (Fig. 4). No diurnal variations except for a slight increase of *phe* transcripts during night times (1.5-fold, $P = 0.05$) were observed.

To assess the influence of the diurnal cycles on mRNA expression, we determined the coefficients of variation (CV) using the normalized gene abundances. If mRNA expression is impacted by the diurnal oscillations in the CW model, the variability between the day and night samples should be greater than the individual variability within the day and within the night samples. The individual day variabilities (median CV, 0.15) were lower than the intersample variability (median CV, 0.29), while the variability among the night samples was similar (CV, 0.30) to intersample variability. For *phe* and *bssA*, the CV in the night samples (CV_{phe} , 0.27; CV_{bssA} , 0.39) were smaller than the intersample CV for these genes (CV_{phe} , 0.30; CV_{bssA} , 0.44), indicating an at most moderate influence of the diurnal cycles on those genes' expression in the CW model. A principal-component analysis also showed a separation of day and night samples based on mRNA expression and abiotic parameters (see Fig. S3 in the supplemental material).

In accordance with the mRNA profiles, protein abundances of the respective toluene degradation enzymes were stable in the day and night samples. Toluene monooxygenase, phenol hydroxylase, and catechol 2,3-dioxygenase showed median $\log_2$ fold changes between 0.1 and -0.03 (Fig. 2 and 3). Previously, we reported that microbial toluene degradation in the CW model is performed aerobically by members of the *Burkholderiales* at low oxygen concentrations (24, 25). During a [¹³C]toluene experiment, their proteins showed >70% ¹³C incorporation, which revealed that toluene was their main but not their sole carbon source. *Ralstonia pickettii* was identified as one of the key players of toluene degradation in the CW model (25). This bacterium showed increased toluene turnover under hypoxic conditions when nitrate was present (40). In this study, many enzymes of toluene degradation matched again to *R. pickettii* or *Hydrogenophaga* sp.

Diurnal root exudate release triggers diurnal regulation of OmpA-like porins and enzymes of amino acid metabolism.

OmpA-like porins were significantly upregulated during the day (median $\log_2$ FC, 0.44; $P = 0.0002$). They have a larger diameter than other porins and therefore allow the transport of complex molecules (37, 41). High-molecular-weight compounds constitute a large portion of root exudates (42), which may lead to increased OmpA-mediated transport during the day (17). In addition, we observed converse trends for enzymes involved in the anabolism of the sulfur-containing amino acids cysteine (cysteine synthase and desulfurase; median $\log_2$ FC = 0.52; $P = 0.014$) and methionine (O-acetylhomoserine sulfhydrylase [OAHs]; median $\log_2$ FC = -0.77 ; $P = 0.013$). The highest fold change was observed for a cysteine synthase belonging to *Rhizobiales* ($\log_2$ FC = 1.75), while the OAHs with the lowest fold change belonged to *Burkholderiales* ($\log_2$ FC = -1.03). Interestingly, ABC transporters assigned to *Rhizobiales* were mainly transporters for sugars, organic acids, and sulfate, while the ABC transporters of the *Burkholderiales* were mostly amino acid transporters. *Rhizobiales* might be more dependent on amino acid synthesis (cysteine synthases), while the *Burkholderiales* take up most amino acids during the day and synthesize methionine during the night (OAHs).

Glutamine synthases were frequently identified in our data set but showed no diurnal trend, except for a glutamine synthase assigned to *Burkholderiales* (GenBank accession number Q63SK2; $\log_2$ FC = 1.4; $P = 0.0097$). This enzyme has a higher affinity for ammonia ($K_m < 0.2$ mM) than glutamate dehydrogenase ($K_m = 3$ mM) and is active when carbon sources are not limited and ammonia concentrations are low (43). In the CW model, there is no apparent carbon limitation, and ammonia concentrations were about 0.7 mM, which triggered the glutamine synthase pathway.

Toluene degraders additionally feed on organic root exudates during the day, regulating fluctuating carbon source availability by diurnal PHA metabolism. While the abundance of most proteins remained stable throughout the diurnal cycle, phasins involved in PHA metabolism were significantly upregulated during the day (median $\log_2$ FC = 1.23; $P < 0.001$). PHA metabolism supports the survival of bacteria living in fluctuating or nutrient-limited environments and has been found in many bacteria isolated from soils and rhizospheres (44). Phasins are amphiphilic proteins which coat PHA granules (45), and the amount of these proteins is correlated with the PHA content of the cell (46). The identified phasins were assigned exclusively to *Burkholderiales*, including the toluene degrader *Ralstonia pickettii*, and 4 of 6 single fold changes were significant, with P values of <0.05 .

Apparently, these bacteria supplement their main carbon and electron source, toluene, during the day with organic plant root exudates, which in turn drives PHA synthesis. A similar diurnal pattern has been found in microbial mats, where heterotrophic microorganisms store excess carbon sources produced and excreted from photosynthetic organisms, such as PHAs, and consume them during night (47). The same mechanism seemed to occur in our wetland model. In addition to phasins being upregulated during the day, specific enzymes of the glyoxylate shunt, malate synthase ($\log_2$ FC = -0.41 ; $P = 0.04$) and isocitrate lyase ($\log_2$ FC = -0.78 ; $P = 0.12$), show increased abundances during the night. Exclusive phylogenetic classification of these enzymes was not possible, but they also matched the enzymes of *R. pickettii* or *Hydrogenophaga*. Bacteria use the glyoxylate shunt to form complex carbohydrates from acetyl-CoA, bypassing the citric acid cycle (48). Apparently, members of the *Burkholderiales* produce PHA during the day and reuse acetyl-CoA from the polymer during the night via the glyoxylate shunt to compensate for the decreased amounts of carbon sources available from the plant. Thus, members of the *Burkholderiales* have an advantage over other bacterial taxa in the CW model. They can use toluene as the main carbon and electron source at low-oxygen concentrations, and additionally, they may cope with the fluctuating conditions in the rhizosphere by modulating their carbon and energy fluxes with PHA metabolism. Acetoacetyl-CoA reductases, which catalyze the reduction of acetoacetyl-CoA to the PHA monomer D-3-hydroxyacetyl-CoA (49), showed no differences during the day and night (median $\log_2$ FC = 0.06). This enzyme seemed to be constitutively expressed, as the respective mRNA showed stable abundances in *Ralstonia eutropha* during normal growth and PHA production (50).

bssA transcripts: no proteomic evidence for anaerobic toluene degradation. Transcripts for the anaerobic enzyme benzylsuccinate synthase (*bssA*) were highly abundant (10^5 copies/ng RNA). Transcript levels were slightly increased during night (1.8-fold; $P = 0.058$) when oxygen concentrations were decreased. Despite the abundance of *bssA* mRNA, no corresponding peptides were detected. Previously, DNA isolated from the CW model showed hybridization with a probe for *bssA* of *Magnetospirillum* sp. strain TS6 (24). In the present study, 23 proteins, including benzoyl-CoA reductase, were phylogenetically assigned to *Magnetospirillum*. As the catalytic activity of benzylsuccinate synthase is irreversibly destroyed by oxygen (51), its absence in the aerobic CW model makes sense; however, a surprise was that *bssA* transcripts were the highest of all quantified genes involved in toluene degradation. The presence of *bssA* transcripts without concomitant *in situ* anaerobic toluene degradation was previously observed (52). Basal expression of *bssA* was induced either by toluene or nitrate, which might explain the detection of *bssA* transcripts without protein translation in our system.

These findings suggest that the sole detection of *bssA* genes and transcripts at a contaminated site can only predict the potential for anaerobic toluene degradation and does not necessarily reflect catabolic activity.

In conclusion, this study illuminates diurnal processes in a CW system and provides insights into polyhydroxyalkanoate metabolism in rhizospheric environments. The model wetland was characterized by fluctuating amounts of oxygen and organic root exudates during the day and night. Aerobic toluene degradation by *Burkholderiales* was stable, while organic plant root exudates were

most probably an additional carbon source during day, which triggered diurnal metabolism of PHA. In addition, transport proteins were significantly upregulated during day, which further indicates a strong influence of diurnal organic exudate release. Despite *bssA* mRNA quantification, anaerobic toluene degradation seemed not to occur in the CW model system. Future studies will focus on the transferability of the results by repeating the investigations in a full-scale CW system treating toluene or benzene-, toluene-, ethylbenzene-, and xylene-contaminated water.

ACKNOWLEDGMENTS

This work contributes to the program topic Chemicals in the Environment (CITE), funded by the Helmholtz Research Programme. The authors are grateful for use of the analytical facilities of the Centre for Chemical Microscopy (ProVIS) at the Helmholtz Centre for Environmental Research, which is supported by European Regional Development Funds (EFRE-Europe funds Saxony) and the Helmholtz Association. Part of this work was funded by the DFG SFB Aquadiva.

Benjamin Scheer (Department of Isotope Biogeochemistry) is acknowledged for technical support for the protein analysis in the ProVIS laboratory. We thank Howard Junca for providing the *bssA*-targeting primers. We thank Kerstin Puschendorf and Claudia Pietsch for anorganic ion and DOC analyses.

REFERENCES

- Moore BJ, Headley JV, Dupont RR, Doucette WD, Armstrong JE. 2002. Abatement of gas-condensate hydrocarbons in a natural wetland. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 37:425–438. <http://dx.doi.org/10.1081/ESE-120003225>.
- Haberl R, Grego S, Langergraber G, Kadlec R, Cicalini A-R, Dias S, Novais J, Aubert S, Gerth A, Thomas H, Hebner A. 2003. Constructed wetlands for the treatment of organic pollutants. *J Soils Sediments* 3:109–124. <http://dx.doi.org/10.1007/BF02991077>.
- Kadlec RH, Wallace SD. 2009. *Treatment wetlands*, 2nd ed. CRC Press, Boca Raton, FL.
- Gessner TP, Kadlec RH, Reaves RP. 2005. Wetland remediation of cyanide and hydrocarbons. *Ecol Eng* 25:457–469. <http://dx.doi.org/10.1016/j.ecoleng.2005.07.015>.
- Wallace S, Kadlec R. 2005. BTEX degradation in a cold-climate wetland system. *Water Sci Technol* 51:165–172.
- Seeger EM, Kusch P, Fazekas H, Grathwohl P, Kästner M. 2011. Bioremediation of benzene-, MTBE- and ammonia-contaminated groundwater with pilot-scale constructed wetlands. *Environ Pollut* 159:3769–3776. <http://dx.doi.org/10.1016/j.envpol.2011.07.019>.
- Bedessem ME, Ferro AM, Hiegel T. 2007. Pilot-scale constructed wetlands for petroleum-contaminated groundwater. *Water Environ Res* 79:581–586. <http://dx.doi.org/10.2175/106143006X111943>.
- Khan S, Afzal M, Iqbal S, Khan QM. 2013. Plant-bacteria partnerships for the remediation of hydrocarbon contaminated soils. *Chemosphere* 90:1317–1332. <http://dx.doi.org/10.1016/j.chemosphere.2012.09.045>.
- Segura A, Ramos JL. 2013. Plant-bacteria interactions in the removal of pollutants. *Curr Opin Biotechnol* 24:467–473. <http://dx.doi.org/10.1016/j.copbio.2012.09.011>.
- Kuiper I, Lagendijk EL, Bloembergen GV, Lugtenberg BJJ. 2004. Rhizoremediation: a beneficial plant-microbe interaction. *Mol Plant Microbe Interact* 17:6–15. <http://dx.doi.org/10.1094/MPMI.2004.17.1.6>.
- Hütsch BW, Augustin J, Merbach W. 2002. Plant rhizodeposition: an important source for carbon turnover in soils. *J Plant Nutr Soil Sci* 165:397–407.
- Nguyen C. 2009. Rhizodeposition of organic C by plant: mechanisms and controls, p 97–123. In Lichtfouse E, Navarrete M, Debaeae P, Véronique S, Alberola C (ed), *Sustainable agriculture*. Springer, Dordrecht, Netherlands.
- Colmer T. 2003. Long-distance transport of gases in plants: a perspective on internal aeration and radial oxygen loss from roots. *Plant Cell Environ* 26:17–36. <http://dx.doi.org/10.1046/j.1365-3040.2003.00846.x>.
- Murray P, Ostle N, Kenny C, Grant H. 2004. Effect of defoliation on patterns of carbon exudation from *Agrostis capillaris*. *J Plant Nutr Soil Sci* 167:487–493. <http://dx.doi.org/10.1002/jpln.200320371>.

15. Waters I, Armstrong W, Thompson C, Setter T, Adkins S, Gibbs J, Greenway H. 1989. Diurnal changes in radial oxygen loss and ethanol metabolism in roots of submerged and nonsubmerged rice seedlings. *New Phytol* 113:439–451. <http://dx.doi.org/10.1111/j.1469-8137.1989.tb00355.x>.
16. Blossfeld S, Gansert D, Thiele B, Kuhn AJ, Lösch R. 2011. The dynamics of oxygen concentration, pH value, and organic acids in the rhizosphere of *Juncus* spp. *Soil Biol Biochem* 43:1186–1197. <http://dx.doi.org/10.1016/j.soilbio.2011.02.007>.
17. Zhai X, Piwpuan N, Arias CA, Headley T, Brix H. 2013. Can root exudates from emergent wetland plants fuel denitrification in subsurface flow constructed wetland systems? *Ecol Eng* 61:555–563. <http://dx.doi.org/10.1016/j.ecoleng.2013.02.014>.
18. Dušek J, Pícek T, Čížková H. 2008. Redox potential dynamics in a horizontal subsurface flow constructed wetland for wastewater treatment: diel, seasonal and spatial fluctuations. *Ecol Eng* 34:223–232. <http://dx.doi.org/10.1016/j.ecoleng.2008.08.008>.
19. Harrison J, Matson P, Fendorf S. 2005. Effects of a diel oxygen cycle on nitrogen transformations and greenhouse gas emissions in a eutrophic subtropical stream. *Aquat Sci* 67:308–315. <http://dx.doi.org/10.1007/s00027-005-0776-3>.
20. Goulet R, Pick F. 2001. Diel changes in iron concentrations in surface-flow constructed wetlands. *Water Sci Technol* 44:421–426.
21. Nikolausz M, Kappelmeyer U, Székely A, Ruzsnyák A, Márialigeti K, Kästner M. 2008. Diurnal redox fluctuation and microbial activity in the rhizosphere of wetland plants. *Eur J Soil Biol* 44:324–333. <http://dx.doi.org/10.1016/j.ejsobi.2008.01.003>.
22. Wiessner A, Kappelmeyer U, Kusch P, Kästner M. 2005. Influence of the redox condition dynamics on the removal efficiency of a laboratory-scale constructed wetland. *Water Res* 39:248–256. <http://dx.doi.org/10.1016/j.watres.2004.08.032>.
23. Kappelmeyer U, Wiefner A, Kusch P, Kästner M. 2002. Operation of a universal test unit for planted soil filters: planted fixed bed reactor. *Eng Life Sci* 2:311–315.
24. Martínez-Lavanchy PM, Chen Z, Lünsmann V, Marin-Cevada V, Vilchez-Vargas R, Pieper DH, Reiche N, Kappelmeyer U, Imperato V, Junca H, Nijenhuis I, Müller JA, Kusch P, Heipieper HJ. 2015. Microbial toluene removal in hypoxic model constructed wetlands occurs predominantly via the ring monooxygenation pathway. *Appl Environ Microbiol* 81:6241–6252. <http://dx.doi.org/10.1128/AEM.01822-15>.
25. Lünsmann V, Kappelmeyer U, Benndorf R, Martínez-Lavanchy PM, Taubert A, Adrian L, Duarte M, Pieper DH, von Bergen M, Müller JA, Heipieper HJ, Jehmlich N. 2016. *In-situ* protein-SIP highlights *Burkholderiaceae* as key players degrading toluene by *para* ring hydroxylation in a constructed wetland model. *Environ Microbiol* 18:1176–1186. <http://dx.doi.org/10.1111/1462-2920.13133>.
26. Duetz WA, de Jong C, Williams PA, van Andel JG. 1994. Competition in chemostat culture between *Pseudomonas* strains that use different pathways for the degradation of toluene. *Appl Environ Microbiol* 60:2858–2863.
27. Leahy JG, Olsen RH. 1997. Kinetics of toluene degradation by toluene-oxidizing bacteria as a function of oxygen concentration, and the effect of nitrate. *FEMS Microbiol Ecol* 23:23–30. <http://dx.doi.org/10.1111/j.1574-6941.1997.tb00387.x>.
28. Chakraborty R, Coates J. 2004. Anaerobic degradation of monoaromatic hydrocarbons. *Appl Microbiol Biotechnol* 64:437–446. <http://dx.doi.org/10.1007/s00253-003-1526-x>.
29. Parales RE, Parales JV, Pelletier DA, Ditty JL. 2008. Diversity of microbial toluene degradation pathways. *Adv Appl Microbiol* 64:1–73. [http://dx.doi.org/10.1016/S0065-2164\(08\)00401-2](http://dx.doi.org/10.1016/S0065-2164(08)00401-2).
30. Faulwetter JL, Gagnon V, Sundberg C, Chazarenc F, Burr MD, Brisson J, Camper AK, Stein OR. 2009. Microbial processes influencing performance of treatment wetlands: a review. *Ecol Eng* 35:987–1004. <http://dx.doi.org/10.1016/j.ecoleng.2008.12.030>.
31. Lund LJ, Horne AJ, Williams AE. 1999. Estimating denitrification in a large constructed wetland using stable nitrogen isotope ratios. *Ecol Eng* 14:67–76. [http://dx.doi.org/10.1016/S0925-8574\(99\)00020-8](http://dx.doi.org/10.1016/S0925-8574(99)00020-8).
32. Shapleigh JP. 2013. Denitrifying prokaryotes, p 405–425. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), *The prokaryotes*. Springer, New York, NY.
33. Vymazal J. 2007. Removal of nutrients in various types of constructed wetlands. *Sci Total Environ* 380:48–65. <http://dx.doi.org/10.1016/j.scitotenv.2006.09.014>.
34. Coban O, Kusch P, Kappelmeyer U, Spott O, Martienssen M, Jetten MS, Knoeller K. 2015. Nitrogen transforming community in a horizontal subsurface-flow constructed wetland. *Water Res* 74:203–212. <http://dx.doi.org/10.1016/j.watres.2015.02.018>.
35. Lacerda CM, Choe LH, Reardon KF. 2007. Metaproteomic analysis of a bacterial community response to cadmium exposure. *J Proteome Res* 6:1145–1152. <http://dx.doi.org/10.1021/pr060477v>.
36. Barr JJ, Dutilh BE, Skennerton CT, Fukushima T, Hastie ML, Gorman JJ, Tyson GW, Bond PL. 2016. Metagenomic and metaproteomic analyses of *Accumulibacter phosphatis*-enriched floccular and granular biofilm. *Environ Microbiol* 18:273–287. <http://dx.doi.org/10.1111/1462-2920.13019>.
37. Nikaido H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* 67:593–656. <http://dx.doi.org/10.1128/MMBR.67.4.593-656.2003>.
38. Thomas AD, Booth IR. 1992. The regulation of expression of the porin gene *ompC* by acid pH. *J Gen Microbiol* 138:1829–1835. <http://dx.doi.org/10.1099/00221287-138-9-1829>.
39. Trautwein K, Kühner S, Wöhlbrand L, Halder T, Kuchta K, Steinbüchel A, Rabus R. 2008. Solvent stress response of the denitrifying bacterium *Aromatoleum aromaticum* strain EbN1. *Appl Environ Microbiol* 74:2267–2274. <http://dx.doi.org/10.1128/AEM.02381-07>.
40. Kukor JJ, Olsen RH. 1996. Catechol 2,3-dioxygenases functional in oxygen-limited (hypoxic) environments. *Appl Environ Microbiol* 62:1728–1740.
41. Bellido F, Martin NL, Siehnell RJ, Hancock RE. 1992. Reevaluation, using intact cells, of the exclusion limit and role of porin OprF in *Pseudomonas aeruginosa* outer membrane permeability. *J Bacteriol* 174:5196–5203.
42. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol* 57:233–266. <http://dx.doi.org/10.1146/annurev.arplant.57.032905.105159>.
43. Helling RB. 1994. Why does *Escherichia coli* have two primary pathways for synthesis of glutamate? *J Bacteriol* 176:4664–4668.
44. Castro-Sowinski S, Burdman S, Matan O, Okon Y. 2010. Natural functions of bacterial polyhydroxyalkanoates, p 39–61. *In* Chen GG-Q (ed), *Plastics from bacteria*, vol 14. Springer, Berlin, Germany.
45. Kuchta K, Chi L, Fuchs H, Pötter M, Steinbüchel A. 2007. Studies on the influence of phasins on accumulation and degradation of PHB and nanostructure of PHB granules in *Ralstonia eutropha* H16. *Biomacromolecules* 8:657–662. <http://dx.doi.org/10.1021/bm060912e>.
46. Pötter M, Madkour MH, Mayer F, Steinbüchel A. 2002. Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16. *Microbiology* 148:2413–2426. <http://dx.doi.org/10.1099/00221287-148-8-2413>.
47. Villanueva L, Navarrete A, Urmeneta J, Geyer R, White DC, Guerrero R. 2007. Monitoring diel variations of physiological status and bacterial diversity in an estuarine microbial mat: an integrated biomarker analysis. *Microb Ecol* 54:523–531. <http://dx.doi.org/10.1007/s00248-007-9224-3>.
48. Dunn MF, Ramirez-Trujillo J, Hernández-Lucas I. 2009. Major roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis. *Microbiology* 155:3166–3175. <http://dx.doi.org/10.1099/mic.0.030858-0>.
49. Anderson AJ, Dawes EA. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol Rev* 54:450–472.
50. Budde CF, Mahan AE, Lu J, Rha C, Sinskey AJ. 2010. Roles of multiple acetoacetyl coenzyme A reductases in polyhydroxybutyrate biosynthesis in *Ralstonia eutropha* H16. *J Bacteriol* 192:5319–5328. <http://dx.doi.org/10.1128/JB.00207-10>.
51. Leuthner B, Leutwein C, Schulz H, Hörth P, Haehnel W, Schiltz E, Schagger H, Heider J. 1998. Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. *Mol Microbiol* 28:615–628. <http://dx.doi.org/10.1046/j.1365-2958.1998.00826.x>.
52. Brow CN, Johnson ROB, Johnson RL, Simon HM. 2013. Assessment of anaerobic toluene biodegradation activity by *bssA* transcript/gene ratios. *Appl Environ Microbiol* 79:5338–5344. <http://dx.doi.org/10.1128/AEM.01031-13>.