Variovorax sp.-mediated biodegradation of the phenyl urea herbicide linuron at micropollutant concentrations and effects of natural dissolved organic matter as supplementary carbon source

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Abstract In nature, pesticides are often present as micropollutants with concentrations too low for efficient biodegradation and growth of heterotrophic pollutant-degrading bacteria. Instead, organic carbon present in environmental dissolved organic matter (eDOM) constitutes the main carbon source in nature. Information on how natural organic carbon affects degradation of pollutants and micropollutants, in particular, is however poor. Linuron-degrading *Variovorax* sp. strains SRS16, WDL1, and PBLH6 and a triple-species bacterial consortium, from which WDL1 originated, were examined for their ability to degrade linuron at micropollutant concentrations and the effect hereon of different eDOM formulations of varying biodegradability as supplementary C-source was explored. Individual strains and the consortium degraded linuron at initial concentrations as low as $1 \mu\text{g L}^{-1}$ till concentrations below 4 ng L^{-1} . Degradation kinetics differed among strains with rates that differed up to 70-fold at the lowest linuron concentrations and with lag phases ranging from 0 to 7 days. Linuron biodegradation by the individual strains was inhibited by an easily biodegradable compound such as citrate but

stimulated by eDOM at a linuron concentration of 10 mg L^{-1} . Effects were strongly reduced or became non-existent at micropollutant linuron concentrations. Effects of eDOM on degradation at 10 mg L^{-1} linuron by WDL1 were reduced when WDL1 was incubated together with its original consortium members. This is the first report on eDOM effects on degradation of pesticides at micropollutant concentrations and indicates these effects are limited and depend on linuron and eDOM concentrations, eDOM quality, and the bacterial culture.

Keywords Biodegradation · Dissolved organic matter · Micropollutants · *Variovorax* · Linuron

Introduction

The ubiquitous use of pesticides has resulted in the contamination of surface and subsurface water bodies. Microbial degradation is considered to be a major route for pesticide removal from the environment. Several heterotrophic bacterial isolates were reported to contain dedicated metabolic pathways that allow them to use specific pesticides as sole sources of carbon and energy and to convert the compounds to harmless and often mineral substituents (Copley 2000). Such strains are regarded as important catalysts to remove pesticides from contaminated water, either through natural attenuation involving indigenous microbial populations or in dedicated treatment systems involving indigenous populations or foreign strains applied by bioaugmentation (Alexander 1981). However, the degradation capacities of such strains have been mainly examined using pesticide concentrations that are far above those encountered in nature. Pesticides in rivers, lakes, aquifers, and wastewater are

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considered micropollutants as they mainly occur at trace amounts, i.e., concentrations ranging from picograms per liter to micrograms per liter (Schwarzenbach et al. 2006). Pesticide concentrations at the milligrams-per-liter level are seldom recorded (Caux et al. 1998). These extreme low micropollutant concentrations still pose a problem for ecological safety and a challenge for developing economical and efficient technologies for drinking water production (Schwarzenbach 2006). In many European countries and in accordance with the European Union Drinking Water Directive 98/83/EC, the limits of an individual active substance in groundwater and drinking water is $0.1 \mu\text{gL}^{-1}$, while the total concentration of all active substances cannot exceed $0.5 \mu\text{gL}^{-1}$. For surface water used as drinking water resource, the total pesticide concentration cannot exceed $5 \mu\text{gL}^{-1}$. The extreme low concentrations at which pesticides occur in groundwater and surface water has been suggested to pose a challenge for their efficient biodegradation (EU 1998) since they allow only minimal growth and be too low for (maximal) induction of or recognition by the catalytic enzymes (Daughton and Ternes 1999; Torng et al. 2003; de Lipthay et al. 2007; Srensen et al. 2007). Moreover, in water, organic carbon present in dissolved organic matter (DOM) and at concentrations that are above those of the pesticide composes the main carbon source for heterotrophic bacteria. The presence of C-sources apart from xenobiotics has been shown to affect xenobiotic degradation either negatively (for instance, by means of catabolic repression) or positively (by generating higher microbial biomass). However, up till now, only few studies report on the capacity of dedicated degraders to remove pesticides and other organic pollutants at micropollutant concentrations (van der Meer et al. 1987; Rapp and Timmis 1999; Srensen 2007). Neither is there information on the impact of the presence of auxiliary C-sources on the degradation of pesticides at micropollutant concentrations.

Recently, Srensen et al. (2009) showed that *Variovorax* sp. strain SRS16, which uses the phenyl urea herbicide linuron as the sole source of carbon and energy, has the ability to mineralize linuron at initial concentrations as low as $2 \mu\text{gL}^{-1}$. The observed degradation of linuron at micropollutant concentrations by strain SRS16 was explained by the constitutive background expression of the catabolic pathway that was sufficient for degrading the compound. This hypothesis was supported by observations made by Bers et al. (2011) which demonstrated background transcription of several genes of the linuron degradation pathway in strain SRS16. One of those genes was *libA* which encodes the amidase responsible for the first step in linuron degradation, i.e., the hydrolysis of linuron into 3,4-dichloroaniline (3,4-DCA). Several other bacterial cultures were reported for their ability to use linuron as sole source of carbon and energy. These cultures include single strains such as *Variovorax* sp. SRS16 but also bacterial

consortia which consist of several member organisms that synergistically degrade linuron. An example of such a consortium is the consortium described by Dejonghe et al. (2003), consisting of *Variovorax* sp. WDL1, *Comamonas testosteroni* WDL7, and *Hyphomicrobium sulfonivorans* WDL6. Interestingly, in all linuron-degrading cultures described up till now, *Variovorax* appears to play a crucial role in linuron degradation by providing the first hydrolysis step. The presence of *Variovorax* as well as that of the *libA* gene has been linked with the natural attenuation of linuron in various ecosystems (Bers 2011; Sniegowski et al. 2011; Bers et al. 2012). The capability of *Variovorax* strains to degrade linuron at micropollutant concentrations, such as recently shown for strain SRS16, is as such of main interest for understanding the fate of linuron in the environment. However, up till now, it is not known whether this capacity extends to other linuron-degrading *Variovorax* sp. strains, including strains which cooperate for linuron degradation in consortia, and how such cooperation affects linuron degradation at micropollutant concentrations. The question whether *Variovorax* strains different from SRS16 are capable of degrading linuron at micropollutant concentrations became recently even more compelling since some linuron-degrading *Variovorax* strains like WDL1 do not contain a *libA* homologue and apparently make use of an alternative linuron amidase (Bers 2011). It remains a question whether the presence of other C-sources affects the degradation of linuron at micropollutant concentrations by *Variovorax* either in isolation or in combination with other strains in the consortium.

The aim of the current study was to determine linuron degradation kinetics at different initial linuron concentrations ranging from 10 mgL^{-1} to $1 \mu\text{gL}^{-1}$ for several linuron-degrading *Variovorax* cultures including single strains and a consortium in order to evaluate their capacity to degrade linuron at micropollutant concentrations and to evaluate consortium effects. In addition, the effect of supplementary carbon sources on linuron degradation at micropollutant concentrations by these cultures was examined by assessing linuron degradation in the presence of DOM of variable quality and quantity. Emphasis was on DOM of environmental origin (eDOM). eDOM was chosen since it composes a relevant and complex mixture of organic compounds in the environment available for growth. This is in contrast with previous two-substrate studies that used pure compounds such as glucose, citrate, etc., at relatively high concentrations (Bally and Egli 1996; Wick et al. 2003). To associate effects of DOM primarily with the organic carbon present in DOM, other macronutrients such as nitrogen, phosphorus, and trace elements were applied at non-limiting concentrations, and a constant pH 7 was maintained. This study provides the first report on the effects of eDOM on the biodegradation of pesticides at environmentally relevant eDOM and pesticide concentrations.

Materials and methods

Chemicals used Pestanal® analytical standards (99.9 %) of linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) and 3,4-dichloroaniline were purchased from Fluka.

Bacterial strains and cultivation Bacterial strains used in this study were *Variovorax* sp. strains WDL1 (LMG27260) (Dejonghe 2003), SRS16 (CIP108393) (Sørensen et al. 2003), and PBLH6 (LMG27259) (Breugelmans et al. 2007); *C. testosteroni* WDL7 (LMG27261) (Dejonghe 2003); and *H. sulfonivorans* WDL6 (LMG27262) (Dejonghe 2003). *Variovorax* sp. strains WDL1, SRS16, and PBLH6 were grown on R2A agar plates amended with 20 mgL⁻¹ linuron for 4 days and subsequently transferred to liquid R2A medium with 20 mgL⁻¹ linuron for 36 h. *C. testosteroni* WDL7 was grown on Luria-Bertani (LB) agar plates for 24 h and colonies transferred to liquid LB medium to grow for 24 h. *H. sulfonivorans* WDL6 was grown on R2A agar plates for 4 days and subsequently grown in liquid minimal medium with nitrogen (MMO) with 1 % methanol for 4–6 days. Bacterial cells of all strains were harvested in late exponential phase and washed repeatedly with 10 mM MgSO₄. Cell densities were adjusted to an OD_{660nm} of 0.1 (approx. 10⁸ cells mL⁻¹). MMO, LB, and R2A media were prepared as described (Dejonghe 2003). Solidified media contained 13 gL⁻¹ agar (Select agar, Invitrogen).

Preparation of DOM stock formulations Trisodiumcitrate was selected as reference DOM, representing an easily degradable carbon substrate. Trisodiumcitrate was dissolved in Milli-Q water at 500 mgL⁻¹ and filter-sterilized (0.22 µm). Three eDOM formulations were used. Humic acids are a recalcitrant fraction of DOM and were isolated from peat soil (Zegveld, The Netherlands) using the extraction method of Levesque and Schnitzer (1967) as described by Buekers (2007). The extracted humic acids were freeze-dried and dissolved in Milli-Q water at 500 mgL⁻¹. DOM recovered from maize leaves leachate was selected to represent allochthonous DOM entering rivers by overland flow and was prepared as reported (Horemans et al. 2012b). River DOM representing surface water DOM originated from the Rourbron spring (Belgium), a freshwater spring located in a peat moor and was prepared as described (Horemans 2012b). All DOM formulations were sequentially filtered over 0.45 and 0.22 µm to retain sterile DOM and stored in an autoclaved glass container (SCHOTT®) at 4 °C. The DOM formulations were regularly checked for stability by measuring dissolved organic carbon (DOC) concentrations with a TOC analyzer (Van Moorlegheem et al. 2011) and for sterility by plate counting on R2A agar plates.

Linuron-degradation experiments In a first experiment, linuron-degradation experiments were performed in the absence of DOM. The experiments were performed in triplicate

in batch in 100-mL Erlenmeyer flasks sealed with cotton plugs and filled with 25 mL of MMO medium containing 10 mgL⁻¹, 1 mgL⁻¹, 100, 10, and 1 µgL⁻¹ linuron. The flasks were inoculated with either one of the *Variovorax* sp. strains or the triple-species consortium consisting of strains WDL1, WDL6, and WDL7 (CONS). In all cases, each strain was added to a final cell density of approximately 10⁶ cells mL⁻¹. A setup consisting of non-inoculated flasks served as a negative abiotic control. Cultures were incubated at 25 °C on a rotary shaker (100 rpm) for 21 days. Samples (600 µL) were taken on daily basis and acidified with 0.3 % (v/v) of a 5 M HCl solution to stop bacterial activity. The samples were centrifuged at 9,391×g for 5 min to remove bacterial cells and stored at -20 °C prior to high-performance liquid chromatography (HPLC) and/or ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis.

A second experiment assessed the effect of DOM on degradation of linuron. The experiments were performed in a similar way as in the first experiment, but DOM was added, and an adjusted MMO medium was used to compensate for the ionic composition of all used DOM formulations. DOC concentrations in the DOM formulations reported above were first adjusted to either 5 or 20 mgL⁻¹ DOC. Ionic compositions were measured as described (Van Moorlegheem 2011). Consequently, taking into account the ionic composition of each DOM formulation, macronutrients and trace elements were added equal to those applied in the minimal medium MMO (Dejonghe 2003) and furthermore adjusted to 1.59 mM NaHCO₃, 1.11 mM NaCl, 0.54 mM Na₂SO₄, and 0.32 mM NaNO₃ to obtain equal ionic composition among all DOM formulations. Afterward, all DOM preparations were filter-sterilized over 0.22 µm, and linuron was added to a final concentration of 10 mgL⁻¹, 100 µgL⁻¹, or 1 µgL⁻¹. Cotton-plug-sealed 100-mL Erlenmeyer flasks containing 25 mL of adjusted MMO with linuron and DOM or adjusted MMO with linuron (as a control) were inoculated in triplicate with either SRS16, WDL1, or the WDL1/WDL6/WDL7 consortium to a final cell density of 10⁶ cells mL⁻¹ of each strain. For each of the tested linuron conditions, one non-inoculated flask was included that served as control for abiotic loss of linuron. Cultures were incubated at 25 °C on a rotary shaker (100 rpm). Samples of 600 µL were taken on daily basis (hourly when needed) and treated for analysis as reported above.

HPLC and UPLC-MS/MS analysis Linuron and 3,4-DCA concentrations in the samples were determined by HPLC in case of concentrations above 10 µgL⁻¹. HPLC analysis was performed with a LaChrom Classic (Hitachi) HPLC system equipped with an Alltima C18 (Alltech®) reversed-phase column of 100 mm length×4.2 mm inner diameter and a particle size of 3 µm. Samples of 20 µL (>100 µgL⁻¹) or

100 μL ($<100 \mu\text{gL}^{-1}$) were injected and eluted with acetonitrile/water (65:35) at 0.8 mL min^{-1} . 3,4-DCA and linuron were detected by UV absorption at 210 nm. Linuron and 3,4-DCA concentrations were determined by using the HSM manager (Hitachi) software. Limit of detection (LOD) and limit of quantification (LOQ) for linuron were respectively 2.4 and $4.1 \mu\text{gL}^{-1}$ and for 3,4-DCA respectively 4 and $5 \mu\text{gL}^{-1}$. UPLC-MS/MS analysis was done in case linuron or 3,4-DCA concentrations were lower than $10 \mu\text{g L}^{-1}$. The LC system consisted of a Thermo Scientific (San José, USA) Accela U-HPLC pumping system, coupled with an Accela Autosampler and Degasser. Chromatographic analysis was achieved by reversed-phase chromatography and gradient elution on a Nucleodur Gravity C18 column ($1.8 \mu\text{m}$, $50 \times 2.1 \text{ mm}$, Macherey-Nagel, PA, USA), kept at 35°C . Injection volume was $100 \mu\text{L}$. Compounds were eluted with a gradient of solvent A (0.01 % (v/v) aqueous formic acid) and solvent B (100 % (v/v) methanol) with the following gradient profile—0–0.5 min hold 60 % B; 0.5–2.0 min from 60 % to 80 % B; 2.0–2.5 min from 80 % to 100 % B; 2.5–4.0 min hold 100 % B, 4.0–4.1 min back to the initial conditions and kept for another minute. Flow rate was $400 \mu\text{L min}^{-1}$. Analysis was performed on a triple quadrupole mass analyzer (TSQ Vantage, Thermo Electron, San José, USA), fitted with a heated electrospray ionization (HESI II) source operating in positive ion mode, using following working conditions—spray voltage at 4 kV; vaporizer and capillary temperature at 350°C and 315°C , respectively; sheath and auxiliary gas at 45 and 5 arbitrary units, respectively; cycle time of 1.5 s. Argon pressure in the collision cell (Q2) was set at 1.5 mTorr, and the mass resolution at the first (Q1) and third (Q3) quadrupoles were set at 0.7 Da at full width at half maximum. S-lens RF amplitude was 63 V for linuron and 67 V for 3,4-DCA. Collision energy in Q2 was respectively 35 eV for transition m/z 249.04>124.99; 33 eV for transition m/z 249.04>132.95; 17 eV for transition m/z 249.04>159.98; and 16 eV for transition m/z 249.04>182.04 of linuron and respectively 41 eV for transition m/z 162.04>64.94; 47 eV for transition m/z 162.04>73.94; 31 eV for transition m/z 162.04>108.94; and 19 eV for transition m/z 162.04>127.01 of 3,4-DCA. Quantification data were acquired based on these four transitions per compound in the selected reaction monitoring mode. Instrument control and data processing were carried out by means of Xcalibur Software 2.0.7 SP1 (Thermo Scientific, San José, USA). LOD and LOQ for linuron were respectively 1 and 4 ngL^{-1} and for 3,4-DCA respectively 50 and 100 ngL^{-1} .

Data analysis Degradation curves as linuron concentrations (micrograms per liter) in function of time (days) were generated for each inoculum in single- and mixed-substrate setups. Curves were fitted with a sigmoidal function by

using SigmaPlot 12.0 (Systat software, Inc., USA) according to Eq. 1.

$$C = \frac{C_0}{1 + e^{\left[\frac{t_{1/2}-t}{b}\right]}} \quad (1)$$

With t as the time (days), $t_{1/2}$ as the time at which linuron was degraded for 50 %, C as the linuron concentration (micrograms per liter), C_0 the linuron concentration at $t=0$, and b a function parameter.

Parameters $t_{1/2}$ and b of the regressions of the degradation curves were estimated for each tested condition and for each inoculant. The lag phase for linuron degradation and the maximal linuron degradation rate were estimated based on the estimates for the parameters and their standard error. For each inoculum, the significant difference in kinetic variables (degradation rate and lag time) between the mixed-substrate and the single-substrate setup was determined with an unpaired Student's t test (significance level 0.05, $n=3$).

Results

Linuron degradation in single-substrate conditions

Degradation of linuron was determined in function of time at initial concentrations of 10,000; 1,000; 100; 10; and $1 \mu\text{gL}^{-1}$ linuron. Recorded linuron degradation curves as well as concomitant accumulation/degradation of 3,4-DCA when linuron was applied at 10 mgL^{-1} are shown in Fig. 1. Accumulated 3,4-DCA was calculated as the ratio of the amount of formed 3,4-DCA over the amount of linuron degraded. At 10 mgL^{-1} linuron, SRS16 showed no lag phase (Fig. 2b) and the highest linuron degradation rate (Fig. 2a) whereas 3,4-DCA accumulation was transient and limited to 3.6 ± 0.07 %. Strain PBLH6 showed a short lag phase of 0.4 ± 0.2 days (Fig. 2b) and a lower linuron degradation rate (Fig. 2a) compared with SRS16 with a similar extent of 3,4-DCA accumulation (4 ± 6 %). Strain WDL1 differed significantly in degradation kinetics compared with strains SRS16 and PBLH6, showing a long lag phase of 6 days (Fig. 2b) and no detectable accumulation of 3,4-DCA. The linuron degradation rate observed for WDL1 (Fig. 2a) was significantly lower than this for SRS16 but was equal to that of PBLH6. Residual linuron concentrations below $1 \mu\text{gL}^{-1}$ were reached by SRS16 and PBLH6 after 1.5 and 5 days, respectively, while WDL1 reached a linuron concentration of around $1 \mu\text{gL}^{-1}$ after 12 days. The lag phase of linuron degradation by the WDL1/WDL6/WDL7 consortium was considerably shortened to 1.5 days compared with WDL1 (Fig. 2b). The linuron degradation rate though remained unchanged (see Fig. 2a). As a consequence, due to the shorter lag time, the time to reach residual linuron concentrations below $1 \mu\text{gL}^{-1}$ was significantly shortened to

Fig. 1 Linuron degradation and 3,4-DCA accumulation/degradation by the tested linuron degrading *Variovorax* sp. strains and consortia at initial linuron concentrations of 10 mg L^{-1} . Residual concentrations of linuron (full line/black symbols, left axis) and accumulated 3,4-DCA (dotted line/white symbols, right axis) for *Variovorax* sp. strains SRS16 (filled inverted triangle), PBLH6 (filled diamond), and WDL1 (filled square) and the consortium of WDL1/WDL6/WDL7 consortium (CONS) (filled circle) are shown. The values are averages from the values obtained with three replicate cultures. Standard deviations are indicated

7 days in the setup inoculated with the consortium compared with the setup inoculated with strain WDL1 separately.

Degradation kinetics changed considerably at lower initial linuron concentrations. The lag phase decreased to 3 to 4 days for WDL1 in setups assessing linuron degradation at an initial linuron concentration of 1 mg L^{-1} compared with the setups assessing linuron degradation at 10 mg L^{-1} . Lag phases were no longer observed below 1 mg L^{-1} linuron for SRS16 and PBLH6 (see Fig. 2b). Also, the lag phase displayed by the

consortium decreased with decreasing initial linuron concentration and disappeared in case of initial linuron concentrations of 10 and 1 mg L^{-1} . In addition, none of the individual strains nor the consortium showed detectable 3,4-DCA accumulation at initial linuron concentrations equal or lower than 1 mg L^{-1} . Recorded linuron degradation rates for the different inocula are shown in Fig. 2a. SRS16 showed significant higher rates over the entire range of used initial linuron concentrations compared with PBLH6 and WDL1, while PBLH6 showed significant

Fig. 2 Recorded linuron degradation rates (a) and lag phases (b) in function of the different initial linuron concentrations for *Variovorax* sp. strains SRS16, PBLH6, and WDL1 and the WDL1/WDL6/WDL7 consortium (CONS). Estimated degradations rates and lag phases (with

95 % confidence intervals) were derived from regression. The values are averages from the values obtained with three replicate cultures. Standard deviations are indicated. Detailed information on the values can be found in Online Resource 1

higher degradation rates compared with WDL1 for initial linuron concentrations of 1 mgL^{-1} and lower. Recorded linuron degradation rates for the triple-species consortium and the individually incubated strain WDL1 were similar for the entire range of tested initial linuron concentrations. After 21 days, all individually inoculated strains, as well as the consortium, reached residual linuron concentrations below 4 ngL^{-1} without detectable accumulation of 3,4-DCA for all initial linuron concentrations.

Effect of DOM presence on linuron degradation The effect of DOM on linuron degradation when DOM was applied as potential supplementary source of carbon and energy besides linuron was studied. In the mixed-substrate setup, SRS16, WDL1, and the WDL1/WDL6/WDL7 consortium were used as inoculum. Linuron degradation in the presence of 5 and 20 mgL^{-1} DOC of DOM was assessed at both macro- and micropollutant concentrations of linuron being 10 mgL^{-1} , $100 \text{ }\mu\text{gL}^{-1}$, and $1 \text{ }\mu\text{gL}^{-1}$. An overview of the results regarding effects of the added DOM formulations on the lag phase before initiation of linuron degradation, on maximal 3,4-DCA accumulation and on linuron degradation rates for SRS16, WDL1, and the WDL1/WDL6/WDL7 consortium, respectively, is provided in Fig. 3 with detailed information on the corresponding values shown in Online Resource 2. No significant abiotic loss of linuron was noted in control systems with and without DOM.

Effects of DOM on linuron degradation by strain SRS16 were observed especially in case of an initial linuron concentration of 10 mgL^{-1} (Fig. 3). Addition of citrate as the supplementary C-substrate resulted in a longer lag phase, a small decrease in the transient 3,4-DCA accumulation, and a higher linuron degradation rate compared with the condition without citrate. These effects were more pronounced at a citrate concentration of 20 mgL^{-1} DOC than at 5 mgL^{-1} DOC. In contrast to citrate, the eDOM formulations (maize leaf leachate, humic acids, and the river DOM) did affect the lag phase positively, i.e., in most cases, the supplementary presence of the eDOM reduced the lag phase both at 5 and 20 mgL^{-1} DOC. In contrast with citrate, in the presence of eDOM, transient 3,4-DCA accumulation decreased with often an increase in the linuron degradation rate at the same time (Fig. 3). Effects of the DOM formulations on linuron degradation at micropollutant concentrations, i.e., at initial concentrations of 100 and $1 \text{ }\mu\text{gL}^{-1}$ linuron were minimal for SRS16 (Fig. 3). Effects were mainly seen on the linuron degradation rate. At $100 \text{ }\mu\text{gL}^{-1}$ linuron, an increase by factor 1.5 of the degradation rate was observed in the presence of humic acids. Transient 3,4-DCA accumulation increased however with 200–900 % in the presence of eDOM. At $1 \text{ }\mu\text{gL}^{-1}$ linuron, addition of maize leaf leachate resulted in a decreased degradation rate by factor 1.5 maximally while the presence of humic acids and river DOM resulted

in an increased degradation rate, however, never exceeding this by factor 1.5.

For WDL1, the clearest effects of DOM were again observed with linuron at a concentration of 10 mgL^{-1} (Fig. 3). The presence of citrate resulted at both DOC concentrations in a lengthening of the lag phase and increased 3,4-DCA accumulation. However, in contrast with SRS16, the linuron degradation rate remained unaffected. The eDOM had primarily a positive effect on linuron degradation by WDL1. For most eDOM, their presence resulted in a shortening of the lag phase and an increasing linuron degradation rate up to a factor 3 with no effect on 3,4-DCA accumulation. At linuron concentrations of 100 and $1 \text{ }\mu\text{gL}^{-1}$, especially, stimulation of the degradation rate was observed, particularly in setups containing eDOM, while the presence of citrate hardly affected linuron degradation. Increases in degradation rates up to factor 5 in the presence of eDOM were recorded. In the presence of eDOM added at 20 mgL^{-1} DOC, WDL1 even achieved linuron degradation rates comparable to that of the WDL1/WDL6/WDL7 consortium, which are higher than this of WDL1 in absence of DOM.

When WDL1 was combined with WDL7 and WDL6 as a consortium, effects of DOM on linuron degradation were quite different from those recorded with WDL1 in isolation (Fig. 3). Effects observed with WDL1 were strongly reduced even at concentrations of 10 mgL^{-1} linuron. At initial linuron concentrations of 10 mgL^{-1} , a reduction of the lag phase was only observed in the presence of maize leaf leachate at both 5 and 20 mgL^{-1} DOC, while none of the DOM, including citrate, affected 3,4-DCA accumulation. Rates were positively affected by all DOM but with factors never exceeding 1.5. In setups containing $100 \text{ }\mu\text{gL}^{-1}$ linuron, limited reduction of the linuron degradation rate was observed with all DOM. In setups containing $1 \text{ }\mu\text{gL}^{-1}$ linuron, some reduction of the linuron degradation rate was recorded in case maize leaf leachate was added.

Discussion

By following the parent compound concentration, all tested linuron-degrading strains, including strain SRS16, demonstrated the ability to degrade linuron at an initial concentration as low as $1 \text{ }\mu\text{gL}^{-1}$ till concentrations below the detection limit of the used analytical method, i.e., 1 ngL^{-1} . Previously, *Variovorax* sp. SRS16 was previously shown to degrade and mineralize linuron at a concentration as low as $2 \text{ }\mu\text{gL}^{-1}$ (Sørensen 2009) by registering $^{14}\text{CO}_2$ production from ^{14}C ring labeled linuron. As such, the different linuron-degrading *Variovorax* strains, although equipped with different initial linuron hydrolysis genes, are capable of degrading linuron at micropollutant concentrations beyond the legal drinking water

Fig. 3 Effects of DOM on linuron degradation by strains SRS16 and WDL1 and the WDL1/WDL6/WDL7 consortium (CONS) at the indicated linuron concentrations. Changes in the linuron degradation rate, lag phase, and 3,4-DCA accumulation in the presence of DOM at 5 and 20 mgL⁻¹ DOC are expressed as percent of the values recorded for the

condition without DOM. The DOM formulations used were citrate (CIT), maize leaf leachate (MLL), humic acids (HA), and river water DOM (ROUR). The values are averages from the values obtained with three replicate cultures. Detailed information on the values can be found in Online Resource 2

limits in the European Union. *Variovorax* strains are hence promising candidates for bioaugmentation of water bodies, water purification systems, and soils contaminated with extremely low linuron concentrations. Biodegradation of organic xenobiotics at the microgram-per-liter level was previously shown but only for a few bacteria and compounds such as dichlobenil (Sørensen 2007) and chlorobenzenes (Rapp 1999). Some bacterial strains showed biodegradation at concentrations at the milligram-per-liter level but not at the microgram-per-liter level for pollutants such as chlorobenzenes (van der Meer 1987) and *p*-nitrophenol (Rubin and Alexander 1983). Since no mineralization was monitored, it is not clear whether degradation of linuron resulted in the production of harmless compounds or its mineral substituents. However, degradation proceeded at all concentrations beyond 3,4-DCA as only transient or no accumulation of 3,4-DCA was observed. Although degradation occurred at micropollutant concentrations of linuron, this does not provide information whether the tested strains grew on linuron. Indeed, at an initial cell density around 10⁶ cells mL⁻¹, sufficient catalysts were probably present for the observed linuron degradation at low concentrations. Despite the common capacity to degrade linuron at micropollutant concentrations, the three strains showed significant differences in degradation rates for all

tested concentrations with SRS16 displaying 30 and 70 times higher rates compared with strains PBLH6 and WDL1, respectively. This might indicate a variability in affinity for linuron between the linuron hydrolases in the different strains. The recently identified linuron hydrolase of strain WDL1 showed a Michaelis–Menten constant K_M that is indeed higher than this of the linuron hydrolase of strain SRS16 (Bers and Springael, unpublished results). Differences in kinetics may also be due to differences in the regulatory and/or uptake system as SRS16 and PBLH6 contain a highly homologous LibA protein (Bers 2011).

Effects of DOM on linuron degradation were especially observed for high linuron concentrations of 10 mgL⁻¹ with the individual strain cultures. Although focus was on eDOM rather than pure and easily degradable compounds, citrate was used as reference DOM, being a tricarboxylic acid cycle intermediate which has been reported to exert carbon catabolite repression (CCR) on pollutant degradation (McFall et al. 1997). Citrate had the most pronounced effects among the different DOM. For SRS16, citrate stalled the initiation of linuron degradation and decreased the accumulation of 3,4-DCA. Apparently, citrate directly exhibits CCR on the expression of the initial linuron degradation step in SRS16. For WDL1, linuron degradation was stalled, but in addition

3,4-DCA accumulated to a greater extent. Therefore, two mechanisms for CCR by citrate on linuron degradation in WDL1 can be proposed. Citrate represses the hydrolase responsible for linuron hydrolysis as well as of the multi-component dioxygenase enzyme responsible for 3,4-DCA conversion. Alternatively, as suggested previously (Breugelmans et al. 2008), citrate exhibited CCR on 3,4-DCA degradation and accumulated 3,4-DCA inhibits linuron hydrolysis (Dejonghe 2003). Since citrate is degraded by both WDL1 and SRS16, the repression on linuron/3,4-DCA degradation is uplifted, and degradation occurs at higher rates due to the increase in population density after growth on citrate (Horemans 2012b), but this was observed for SRS16 and not for WDL1.

Effects of eDOM formulations were greatest at 10 mgL^{-1} linuron in pure culture systems. In contrast with citrate, for both SRS16 and WDL1, eDOM stimulated linuron degradation at 10 mgL^{-1} linuron. Environmental DOM consists of a complex combination of various organic compounds each present at concentrations at the microgram-per-liter and nanogram-per-liter levels (Kovárová-kovar and Egli 1998). We hypothesize that compounds in eDOM, exerting CCR on pollutant degradation such as specific carboxylic acids and carbohydrates (McFall 1997, Rentz et al. 2004), are present at concentrations too low for affecting linuron degradation. Similarly, Keuth and Rehm (1991) observed the repression of phenanthrene metabolism by *Arthrobacter polychromogenes* in the presence of 4 gL^{-1} glucose but not at 0.45 gL^{-1} . In addition, as proposed by Kovárová-kovar (1998), mixed C-sources at low concentrations might be used simultaneously and will lead to growth and increased population sizes hereby reducing lag times and increasing degradation rates (Schmidt and Alexander 1985; Hess et al. 1990; Wick 2003). Biodegradation and growth of strains SRS16, PBLH6, WDL1, as well as the consortium on the three eDOM formulations used in this study was indeed observed (Horemans et al. 2012a, 2012b). The increase in linuron degradation rates and especially the dramatic decrease in lag phase (−80 %) that was recorded for WDL1, however, cannot be fully explained by supplementary growth. This is particularly the case with river DOM (ROUR) which was found to be the least biodegradable of the three tested eDOM formulations and which did result in a lower growth rate or yield compared with maize leaf leachate eDOM (Horemans 2012b). A possible explanation is that specific (aromatic) compounds present in eDOM induce linuron catabolic enzymes and stimulate linuron degradation. Previously, Larsson et al. (1988) showed that chlorinated non-target compounds led to enhanced degradation of a chlorinated target compound by inducing the corresponding catabolic pathway. An alternative explanation is that biodegradation was enhanced in the presence of the river eDOM by increasing linuron uptake after formation of DOM–linuron complexes as observed for the biodegradation

of the polycyclic aromatic hydrocarbon phenanthrene in the presence of humic acids (Ortega-Calvo and Saiz-Jimenez 1998; Smith et al. 2009). The complexation of organic pollutants with DOM depends on the partitioning coefficient between water and DOM (k_{DOC}) and the amount of DOM and its quality (Chiou et al. 1986, Iglesias-Jiménez et al. 1997). The relatively low $\log k_{\text{DOC}}$ values for linuron (i.e., 2.37–3.02) (Delle Site 2001) implies that only 0.5 % to 2.0 % of linuron will be reversibly associated with DOM and will likely not affect biodegradation kinetics unless other forces than hydrophobic forces determine complexation. The degradation of 10 mgL^{-1} linuron in the presence of eDOM was especially enhanced in case of WDL1, while effects were rather minimal in case of SRS16 and can be explained by the time frame in which linuron was degraded. SRS16 degrades linuron in the order of hours and WDL1 in the order of days. Full growth on eDOM is rather slow (14 days) for both SRS16 and WDL1. As a consequence, SRS16 is expected to degrade linuron fully prior to growth on DOM while for WDL1, the use of eDOM and linuron will be a simultaneous process.

Citrate and eDOM impacted lag phase and degradation rates for WDL1 and SRS16 to no or lesser extent at low concentrations ($<100 \text{ }\mu\text{gL}^{-1}$) compared with 10 mgL^{-1} linuron. Effects of citrate were positive rather than negative, suggesting that CCR observed with high linuron concentrations are no longer applicable at lower citrate concentrations. Other authors previously observed carbon regulation control mechanisms at high pollutant concentrations that ceased to have an effect below certain pollutant concentrations (Müller et al. 1996; McFall 1997). Previously, it was suggested that linuron degradation by SRS16 at micropollutant concentrations is due to background expression of the involved catabolic genes (Sørensen 2009; Bers 2011), and apparently, this background expression is not affected by DOM. The increased linuron-degradation rates in the presence of citrate might then be explained by the increased population size due to growth on citrate. In contrast to high linuron concentrations, where eDOM yielded overall positive effects on degradation, at 100 and $1 \text{ }\mu\text{gL}^{-1}$ linuron a minimal effect was noted despite the anticipated increase in population size and/or enzymes and/or increased expression of linuron degradation genes as discussed above. Likely, at a linuron concentration at the microgram-per-liter level, the increase in biomass is irrelevant to increase in the activity.

Interestingly, the effects of citrate and eDOM observed with WDL1 were strongly reduced when WDL1 was combined with WDL7 and WDL6, recomposing the linuron-degrading consortium. Apparently, combining WDL1 with other strains buffered against both positive and negative effects of DOM on linuron degradation by WDL1. The other consortium members also degrade citrate and compounds in eDOM, hereby mitigating effects they likely have on WDL1 as C-source or inducer/repressor of linuron degradation.

Increased metabolic activity toward single C-substrates as well as eDOM was observed with the consortium compared with the individual strains (Horemans et al. 2012a; Horemans 2012b). WDL7 probably is of main importance in this as *Comamonas* spp. have wide metabolic capacities while *Hyphomicrobium* spp. primarily use C1-compounds (Horemans 2012a). The importance of the diversity within microbial communities to protect pollutant degraders from antimicrobial compounds or CRC exerting compounds by biodegradation has been previously suggested by Wiggins and Alexander (1988)).

In conclusion, linuron-degrading *Variovorax* sp. strains display an overall ability to degrade linuron at the microgram-per-liter level beyond a residual concentration of 4 ngL⁻¹. This ability was never fully impeded in the presence of uncharacterized DOM as supplementary C-source, while it did affect the linuron-degrading activity at high linuron concentrations. The integration of linuron degraders in a microbial consortium reduces effects exerted on the linuron degrader as an individual. As such, effects on linuron degradation depend on (1) the *Variovorax* sp. strain, (2) the applied linuron concentration, (3) the applied DOM, and (4) the co-presence of other strains.

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References

- Alexander M (1981) Biodegradation of chemicals of environmental concern. *Science* 211:132–138. doi:10.1126/science.7444456
- Bally M, Egli T (1996) Dynamics of substrate consumption and enzyme synthesis in *Chelatobacter heintzii* during growth in carbon-limited continuous culture with different mixtures of glucose and nitrilotriacetate. *Appl Environ Microbiol* 62:133–140
- Bers K, Sniegowski K, De Mot R, Springael D (2012) Dynamics of the linuron hydrolase libA gene pool size in response to linuron application and environmental perturbations in agricultural soil and on-farm biopurification systems. *Appl Environ Microbiol* 78:2783–2789. doi:10.1128/aem.06991-11
- Bers K, Leroy B, Breugelmans P, Albers P, Lavigne R, Sørensen S, Springael D (2011) A novel linuron hydrolase identified by genomic-proteomic analysis of phenylurea herbicide mineralization by *Variovorax* sp. strain SRS16. *Appl Environ Microbiol* 78. doi:10.1128/AEM.06162-11
- Breugelmans P, D'Huys P-J, De Mot R, Springael D (2007) Characterization of novel linuron-mineralizing bacterial consortia enriched from long-term linuron-treated agricultural soils. *FEMS Microbiol Ecol* 62:374–385. doi:10.1111/j.1574-6941.2007.00391.x
- Breugelmans P, Barken KB, Tolker-Nielsen T, Hofkens J, Dejonghe W, Springael D (2008) Architecture and spatial organization in a triple-species bacterial biofilm synergistically degrading the phenylurea herbicide linuron. *FEMS Microbiol Ecol* 64:271–282. doi:10.1111/j.1574-6941.2008.00470.x
- Buekers J (2007) Fixation of cadmium, copper, nickel and zinc in soil: kinetics, mechanism and its effect on metal bioavailability. Dissertation, Catholic University of Leuven, Leuven, Belgium
- Caux PY, Kent RA, Fan GT, Grande C (1998) Canadian water quality guidelines for linuron. *Environ Toxicol and Water Quality* 13:1–41. doi:10.1002/(sici)1098-2256(1998)13:1<1::aid-tox1>3.0.co;2-b
- Chiou CT, Malcolm RL, Brinton TI, Kile DE (1986) Water solubility enhancement of some organic pollutants and pesticides by dissolved humic and fulvic acids. *Environ Sci & Tech* 20:502–508. doi:10.1021/es00147a010
- Copley S (2000) Evolution of a metabolic pathway for degradation of a toxic xenobiotic: the patchwork approach. *Trends Biochem Sci* 25:261–265. doi:10.1016/S0968-0004(00)01562-0
- Daughton CG, Ternes TA (1999) Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ Health Perspect* 107:907–938. doi:10.1016/j.envpol.2005.12.018
- de Liphay JR, Sørensen SR, Aamand J (2007) Effect of herbicide concentration and organic and inorganic nutrient amendment on the mineralization of mecoprop, 2,4-D and 2,4,5-T in soil and aquifer samples. *Environ Pollut* 148:83–93. doi:10.1016/j.envpol.2006.11.005
- Dejonghe W, Berteloot E, Goris J, Boon N, Crul K, Maertens S, Höfte M, Vos PD, Verstraete W, Top EM (2003) Synergistic degradation of linuron by a bacterial consortium and isolation of a single linuron-degrading *Variovorax* strain. *Appl Environ Microbiol* 69:1532–1541. doi:10.1128/AEM.69.3.1532-1541.2003
- Delle Site A (2001) Factors affecting sorption of organic compounds in natural sorbent/water systems and sorption coefficients for selected pollutants. A review. *J Phys Chem Ref Data* 30:187–439
- EU (1998) Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Council of the European Union
- Hess TF, Schmidt SK, Silverstein J, Howe B (1990) Supplemental substrate enhancement of 2,4-dinitrophenol mineralization by a bacterial consortium. *Appl Environ Microbiol* 56:1551–1558
- Horemans B, Smolders E, Springael D (2012a) Carbon source utilization profiles suggest additional metabolic interactions in a synergistic linuron degrading bacterial consortium. *FEMS Microbiol Ecol*. doi:10.1111/1574-6941.12033
- Horemans B, Vandermaesen J, Smolders E, Springael D (2012b) Cooperative dissolved organic carbon assimilation by a linuron degrading bacterial consortium. *FEMS Microbiol Ecol*. doi:10.1111/1574-6941.12036
- Iglesias-Jiménez E, Poveda E, Sánchez-Martín MJ, Sánchez-Camazano M (1997) Effect of the nature of exogenous organic matter on pesticide sorption by the soil. *Archives of Environ Contamination and Toxicol* 33:117–124. doi:10.1007/s002449900232
- Keuth S, Rehm H-J (1991) Biodegradation of phenanthrene by *Arthrobacter polychromogenes* isolated from a contaminated soil. *Appl Microbiol Biotechnol* 34:804–808. doi:10.1007/bf00169354
- Kovárová-kovar K, Egli T (1998) Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. *Microbiol Mol Biol Rev* 62:646–666
- Larsson P, Okla L, Tranvik L (1988) Microbial degradation of xenobiotic, aromatic pollutants in humic water. *Appl Environ Microbiol* 54:1864–1867
- Levesque M, Schnitzer M (1967) The extraction of soil organic matter by base and chelating resin. *Can J Soil Sci* 25:47–51. doi:10.4141/cjss67-012
- McFall SM, Abraham B, Narsolis CG, Chakrabarty AM (1997) A tricarboxylic acid cycle intermediate regulating transcription of a chloroaromatic biodegradative pathway: fumarate-mediated repression of the *clcABD* operon. *J Bacteriol* 179:6729–6735

- Müller C, Petruschka L, Cuypers H, Burchhardt G, Herrmann H (1996) Carbon catabolite repression of phenol degradation in *Pseudomonas putida* is mediated by the inhibition of the activator protein *PhlR*. *J Bacteriol* 178:2030–2036
- Ortega-Calvo JJ, Saiz-Jimenez C (1998) Effect of humic fractions and clay on biodegradation of phenanthrene by a *Pseudomonas fluorescens* strain isolated from soil. *Appl Environ Microbiol* 64:3123–3126
- Rapp P, Timmis KN (1999) Degradation of chlorobenzenes at nanomolar concentrations by *Burkholderia* sp. strain PS14 in liquid cultures and in soil. *Appl Environ Microbiol* 65:2547–2552
- Rentz JA, Alvarez PJJ, Schnoor JL (2004) Repression of *Pseudomonas putida* phenanthrene degrading activity by plant root extracts and exudates. *Environ Microbiol* 6:574–583. doi:10.1111/j.1462-2920.2004.00589.x
- Rubin HE, Alexander M (1983) Effect of nutrients on the rates of mineralisation of trace concentrations of phenol and p-nitrophenol. *Environ Sci Technol* 17:104–107. doi:10.1021/es00108a008
- Schmidt SK, Alexander M (1985) Effects of dissolved organic carbon and second substrates on the biodegradation of organic compounds at low concentrations. *Appl Environ Microbiol* 49:822–827
- Schwarzenbach R, Escher BI, Fenner K, Hofstetter TB, Johnson CA, von Gunten U, Wehrli B (2006) The challenge of micropollutants in aquatic systems. *Science* 313:1072–1077. doi:10.1126/science.1127291
- Smith KEC, Thullner M, Wick LY, Harms H (2009) Sorption to humic acids enhances polycyclic aromatic hydrocarbon biodegradation. *Environ Sci Technol* 43:7205–7211. doi:10.1021/es803661s
- Sniegowski K, Bers K, Van Goetem K, Ryckeboer J, Jaeken P, Spanoghe P, Springael D (2011) Improvement of pesticide mineralization in on-farm biopurification systems by bioaugmentation with pesticide-primed soil. *FEMS Microbiol Ecol* 76:64–73. doi:10.1111/j.1574-6941.2010.01031.x
- Sørensen SR, Simonsen A, Aamand J (2009) Constitutive mineralization of low concentrations of the herbicide linuron by a *Variovorax* sp. strain. *FEMS Microbiol Lett* 292:291–296. doi:10.1111/j.1574-6968.2009.01501.x
- Sørensen SR, Holtze MS, Simonsen A, Aamand J (2007) Degradation and mineralization of nanomolar concentrations of the herbicide dichlobenil and its persistent metabolite 2,6-dichlorobenzamide by *Aminobacter* spp. isolated from dichlobenil-treated soils. *Appl Environ Microbiol* 73:399–406. doi:10.1128/aem.01498-06
- Sørensen SR, Bending GD, Jacobsen CS, Walker A, Aamand J (2003) Microbial degradation of isoproturon and related phenylurea herbicides in and below agricultural fields. *FEMS Microbiol Ecol* 45:1–11. doi:10.1016/S0168-6496(03)00127-2
- Toräng L, Nyholm N, Albrechtsen H-J (2003) Shifts in biodegradation kinetics of the herbicides MCPP and 2,4-D at low concentrations in aerobic aquifer materials. *Environ Sci Technol* 37:3095–3103. doi:10.1021/es026307a
- van der Meer JR, Roelofs W, Schraa G, Zehnder AJB (1987) Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 in non-sterile soil columns. *FEMS Microbiol Lett* 45:333–341. doi:10.1111/j.1574-6968.1987.tb02410.x
- Van Moorleghem C, Six L, Degryse F, Smolders E, Merckx R (2011) Effect of organic P forms and P present in inorganic colloids on the determination of dissolved P in environmental samples by the diffusive gradient in thin films technique, ion chromatography, and colorimetry. *Anal Chem* 83:5317–5323. doi:10.1021/ac200748e
- Wick LY, Pasche N, Bernasconi SM, Pelz O, Harms H (2003) Characterization of multiple-substrate utilization by anthracene-degrading *Mycobacterium frederiksbergense* LB501T. *Appl Environ Microbiol* 69:6133–6142. doi:10.1128/AEM.69.10.6133-6142.2003
- Wiggins BA, Alexander M (1988) Role of chemical concentration and second carbon sources in acclimation of microbial communities for biodegradation. *Appl Environ Microbiol* 54:2803–2807