

RESEARCH ARTICLE

Open Access



Delftia sp. LCW, a strain isolated from a constructed wetland shows novel properties for dimethylphenol isomers degradation

Mónica A. Vásquez-Piñeros¹, Paula M. Martínez-Lavanchy^{1,2}, Nico Jehmlich³, Dietmar H. Pieper⁴, Carlos A. Rincón¹, Hauke Harms⁵, Howard Junca⁶ and Hermann J. Heipieper^{1*} 

Abstract

Background: Dimethylphenols (DMP) are toxic compounds with high environmental mobility in water and one of the main constituents of effluents from petro- and carbochemical industry. Over the last few decades, the use of constructed wetlands (CW) has been extended from domestic to industrial wastewater treatments, including petro-carbochemical effluents. In these systems, the main role during the transformation and mineralization of organic pollutants is played by microorganisms. Therefore, understanding the bacterial degradation processes of isolated strains from CWs is an important approach to further improvements of biodegradation processes in these treatment systems.

Results: In this study, bacterial isolation from a pilot scale constructed wetland fed with phenols led to the identification of *Delftia* sp. LCW as a DMP degrading strain. The strain was able to use the *o*-xlenols 3,4-DMP and 2,3-DMP as sole carbon and energy sources. In addition, 3,4-DMP provided as a co-substrate had an effect on the transformation of other four DMP isomers. Based on the detection of the genes, proteins, and the inferred phylogenetic relationships of the detected genes with other reported functional proteins, we found that the phenol hydroxylase of *Delftia* sp. LCW is induced by 3,4-DMP and it is responsible for the first oxidation of the aromatic ring of 3,4-, 2,3-, 2,4-, 2,5- and 3,5-DMP. The enzyme may also catalyze both monooxygenation reactions during the degradation of benzene. Proteome data led to the identification of catechol *meta* cleavage pathway enzymes during the growth on *ortho* DMP, and validated that cleavage of the aromatic rings of 2,5- and 3,5-DMPs does not result in mineralization. In addition, the tolerance of the strain to high concentrations of DMP, especially to 3,4-DMP was higher than that of other reported microorganisms from activated sludge treating phenols.

Conclusions: LCW strain was able to degraded complex aromatics compounds. DMPs and benzene are reported for the first time to be degraded by a member of *Delftia* genus. In addition, LCW degraded DMPs with a first oxidation of the aromatic rings by a phenol hydroxylase, followed by a further *meta* cleavage pathway. The higher resistance to DMP toxicity, the ability to degrade and transform DMP isomers and the origin as a rhizosphere bacterium from wastewater systems, make LCW a suitable candidate to be used in bioremediation of complex DMP mixtures in CWs systems.

Keywords: Xlenols, Biodegradation, Phenol hydroxylase, Toxicity, Constructed wetlands, *Delftia* sp.

* Correspondence: hermann.heipieper@ufz.de

¹Helmholtz Centre for Environmental Research – UFZ, Department of Environmental Biotechnology, Permoserstr. 15, Leipzig, Germany
Full list of author information is available at the end of the article



Background

It is well known that the wastewater of coal conversion and coke operation processes contains high concentrations of phenolic compounds, constituting up to 80% of the total chemical oxygen demand (COD) [1]. The phenolic fraction of the this wastewater is mainly represented by phenol, cresols, resorcinols and dimethylphenols (DMP) [2], substances which are considered toxic, carcinogenic, mutagenic and teratogenic [3]. The DMPs, have shown to be even more persistent and toxic than other phenolic compounds [4] and they are often released to the environment in higher concentration than the permissible limits established [1, 5].

Biological treatment of phenols and their derivatives has been the main approach for their removal [6, 7]. Biodegradation of the six DMP isomers, in pure or mixed cultures, often involves different *Pseudomonas* species [8–10]. Degradation of 2,6-DMP has only been reported by *Mycobacterium* sp. strain DM1 [11]. Studies aiming at the removal of DMP in a mixture with other substituted phenols in laboratory-scale sludge units have also been performed [6, 12]. Recently, constructed wetlands (CWs) have been investigated for their potential to remove coke oven wastewater [13]. Promisingly, Schultze-Nobre et al., reported efficient removal of a mixture of three DMP-isomers (2,6-, 3,4- and 3,5-DMP) in a laboratory scale CW [14].

Complex physicochemical and biological processes in CWs include the interaction of plants, microorganism and pollutants. The main role in the transformation and mineralization of organic pollutants is played by microorganisms present in the rhizosphere, an environment suitable for highly efficient transformations of complex contaminants [15, 16]. There is, however, little knowledge about the bacterial removal of DMP in CWs and detailed microbiological and molecular biological studies

are needed to reveal the biological activities towards DMP as a basis for better management of CWs.

In this work, we aimed to isolate strains from a constructed wetland treating phenolic wastewater in order to characterize the ability to degrade DMP isomers, the toxicity tolerance toward the isomers and to reveal the genomic and proteomic mechanisms supporting the degradation. The first results indicated that *Delftia* sp. LCW is a versatile bacterium with novel properties to degrade DMP isomers and it could be used to enhance phenolic compounds removal in CWs.

Results

DMP-degrading bacterium, growth and co-metabolism assays

From the isolation trials, LCW was the strain recovered with capabilities to use DMP as sole carbon and energy source. According to its 16S rRNA gene sequence, the isolate is a member of the genus *Delftia*. The 16S rRNA gene is identical to that of *Delftia* sp. strain SM-1 (JN001163) and differs by only 1–2 bases from that of various *Delftia acidovorans* isolates (e.g. *D. acidovorans* SPH-1, CP000884 and *D. acidovorans* NBRC 14950, AB680719; 99.9% sequence identity) but by 7–8 bases from that of *Delftia tsuruhatensis* and *Delftia lacustris* strains, including the respective type strains (*D. lacustris* 332 EU888308; *D. tsuruhatensis* T7, AB075017).

The strain was able to use 2,3-DMP and 3,4-DMP as sole carbon and energy source (Fig. 1a and b). Complete depletion of the isomers was observed and both substrates yielded similar bacterial biomass (Table 1).

In mixtures of 3,4- and 2,3-DMP the latter isomer sharply decreased the lag phase (Fig. 2a). *Delftia* sp. LCW was not able to grow with the other four DMP isomers. However, in the presence of 3,4-DMP, the isomers 2,5- and

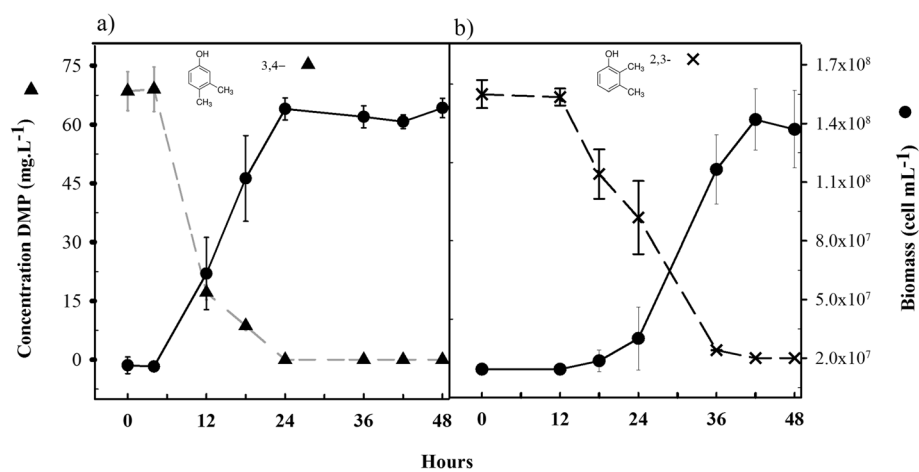


Fig. 1 Growth (circle) of *Delftia* sp. LCW and degradation of **a** 3,4-DMP (triangle) and **b** 2,3-DMP (cross) as sole carbon and energy source. Bars represent \pm SD

Table 1 Yield coefficients and bacterial biomass for *Delftia* sp. LCW grown on DMP-isomers. The overall initial concentration of the DMP in all treatments was 70 mg L⁻¹

DMP	Maximal Biomass (cell mL ⁻¹)	Yield (mg dry weight mg ⁻¹ C-DMP)
3,4-	1.48 × 10 ⁸ ± 5.66 × 10 ⁶	0.73 ± 0.04
2,3-	1.42 × 10 ⁸ ± 1.56 × 10 ⁸	0.69 ± 0.62
3,4- + 2,3-	1.49 × 10 ⁸ ± 1.27 × 10 ⁷	0.73 ± 0.07
3,4- + 3,5-	7.88 × 10 ⁷ ± 4.10 × 10 ⁶	0.28 ± 0.04
3,4- + 2,5	8.61 × 10 ⁷ ± 1.01 × 10 ⁷	0.39 ± 0.02
3,4- + 2,4	6.48 × 10 ⁷ ± 1.54 × 10 ⁷	0.35 ± 0.02
3,4- + 2,6	6.24 × 10 ⁷ ± 1.82 × 10 ⁶	0.30 ± 0.66

3,5-DMP were completely transformed (Fig. 2b and c), whereas partial transformation of 2,4-DMP (Fig. 2d) and no transformation of 2,6-DMP were observed (Fig. 2e). The mixture of 3,4- and 2,3- DMP gave rise to a similar yield to the one obtained with 3,4-DMP alone (Table 1). Meanwhile, mixtures with 3,4- plus 3,5-, 2,5- or 2,4-DMP yielded approx. half of the bacterial yield (Table 1). In addition, bacterial biomass was significantly higher in the mixture of 3,4- and 2,3- DMP than the biomass of the other DMP mixtures (*p* < 0.001, Additional file 1: Table A). Therefore, it was evident that none cell growth was giving by 3,5-, 2,5- and 2,4-DMP. No abiotic losses were detected in any DMP isomer solutions, proving that the decrease in DMP concentrations was due to microbial activity (data not shown).

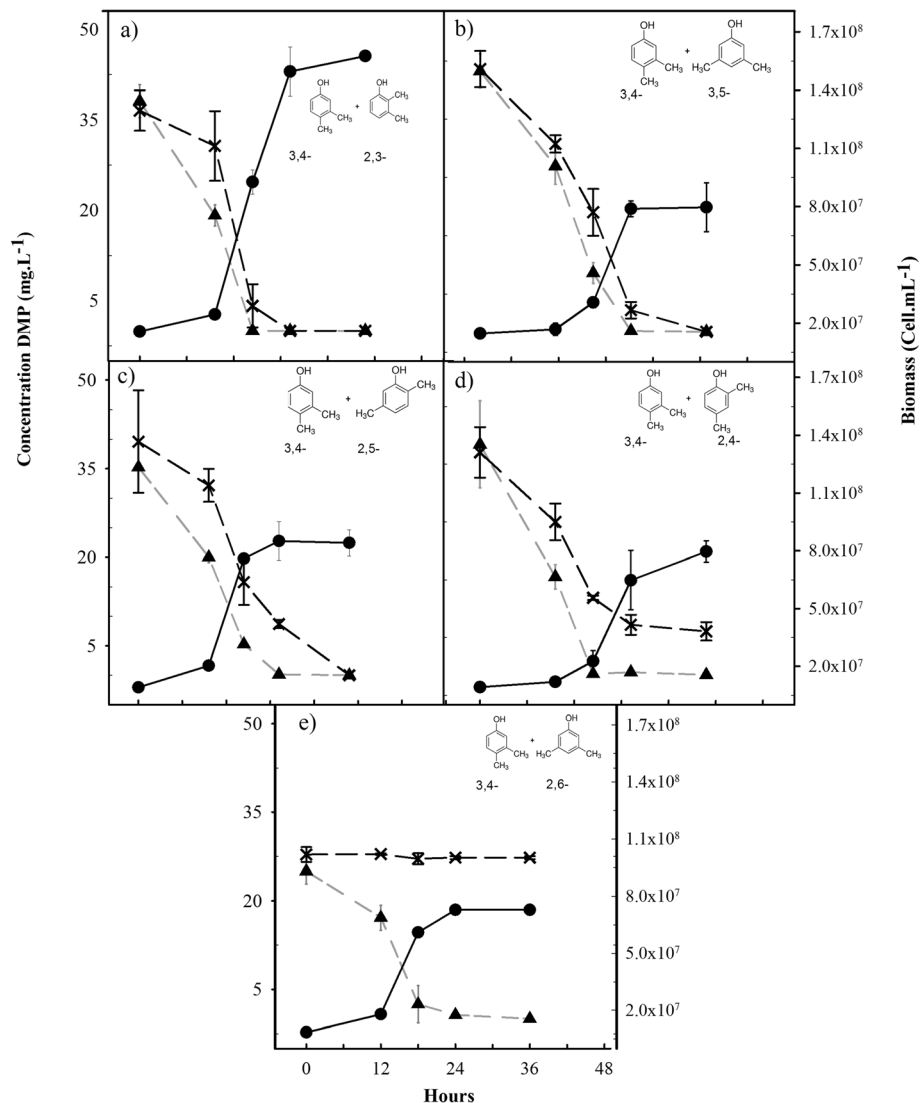


Fig. 2 Growth (circle) of *Delftia* sp. LCW and degradation of the isomeric mixtures. **a** 3,4-DMP (triangle) and 2,3-DMP (cross), **b** 3,4-DMP (triangle) with 3,5-DMP (cross), **c** 3,4-DMP (triangle) with 2,5-DMP (cross), **d** 3,4-DMP (triangle) with 2,4-DMP (cross), and **e** 3,4-DMP (triangle) with 2,6-DMP (cross). Bars represent ±SD

Detection of aerobic catabolic genes in *Delftia* sp. LCW

Among the different primer sets targeting catabolic genes tested with *Delftia* sp. LCW, amplification was only observed with phenol hydroxylase gene primers. The deduced protein sequence had a length of 214 amino-acids and the most closely related identified protein was the phenol hydroxylase large subunit from *Delftia* sp. AZ1-13 (ACZ44761). The protein clustered with phenol hydroxylases of *Comamonas testosteroni* TA441 (BAA34172) and other members of the family Comamonaceae, as well as some few toluene monooxygenases of *Burkholderia cepacia* (strain JS150-AAG40791 and strain G4-AAL50373) (Fig. 3). It was not possible to detect amplification of other BTEX catabolic genes with the set of primers used.

Growth of *Delftia* sp LCW. on other aromatics

D. acidovorans grew on 30 mg L⁻¹ benzene to a biomass of $7.44 \times 10^7 \pm 1.46 \times 10$ cell x mL within 48 h. Growth of the strain on other BTEX compounds was not observed.

Proteomic analysis

The label-free shotgun proteomics approach allowed the identification of 1814 proteins (Additional file 2). First, an unsupervised clustering approach (Principal Component Analysis, PCA) based on the different treatments, revealed clear segregation by sample treatment. The variances among the treatment groups showed that replicate analyses are clustering together. The PCA analysis also showed a greater segregation between the proteome profile of 3,4-DMP and 2,3-DMP among the four treatments (Additional file 3). The proteins that were significantly different between 3,4- and 2,3-DMP, were functionally assigned according to KO (KEEG Orthology), which characterizes gene functions in order to infer high level protein functions of the organism. The main functional categories of the proteins that differed between 3,4- and 2,3-DMP treatments can be found in Additional file 4.

In total, five proteins related with aromatic degradation pathways could be identified (Table 2). Phenol hydroxylase, catechol 2,3-dioxygenase, 2-hydroxymuconic-semialdehyde dehydrogenase and 4-oxalocronate tautomerase were identified in all treatments. Protocatechuate 4,5-dioxygenase was identified only in 2,3-DMP grown cells. In Fig. 4, the protein abundance of related DMP proteins present in 2,3- and 3,4-DMP as singles isomers are indicated. There were not significant differences of the abundances of the proteins between for 2,3- and 3,4-DMP treatments, for any of the proteins related with aromatic degradation (phenol hydroxylase, $p = 0.160$; catechol 2,3-dioxygenase, $p = 0.227$, 2-hydroxymuconic semialdehyde dehydrogenase, $p = 0.488$ and 4-oxalocronate tautomerase $p = 0.227$) (Additional file 1: Table B).

Toxicity of DMP-isomers to *Delftia* sp. LCW

Pearson correlation coefficient between EC₅₀ and the log P_{ow} showed a negative correlation for all DMPs-isomers (correlation coefficient -0.978 and p -value =0.0007). Hence, 3,5-DMP (represented by the highest log P_{ow}) had the lowest EC₅₀ value, followed by 2,4-, 2,3-, 2,6-, 2,5- and 3,4-DMP (Fig. 5).

Discussion

Based on the best performance to degrade DMP among the obtained isolates, the strain named as *Delftia* sp. LCW was selected. This isolate proved to be a close relative of the type strain *Delftia* sp. SM-1 and several strains of *Delftia acidovorans*. Strains of *D. acidovorans*, formerly known as *Comamonas acidovorans* [17], have been reported as degraders of 4-nitrobenzoate [18], 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MPCA) [19]. Other isolates from the genus *Delftia* are able to degrade a wide range of phenoxyalkanoic acid herbicides, chlorinated phenols [20–22], sodium dodecyl sulfate [23], phenanthrene [24] and taurocholate [25]. Another family related bacterium, *Comamonas testosteroni* CNB-2 has been also reported to degrade 4-chlorophenol (4-CP), phenol and methylphenols [26, 27]. However, to our knowledge this is the first report of a *Delftia* strain able to degrade DMP and benzene.

Delftia sp. LCW was able to grow on the *ortho*-DMP isomers as a sole carbon and energy source. A similar growth profile has previously reported for *Cupriavidus pinatobuensis* JMP 134 [28], *Pseudomonas* CF600 [10, 29], *Pseudomonas aeruginosa* T1 [30] and *Comamonas testosteroni* JH5 [26].

In general, DMP biodegradation have been reported for all isomers, mainly through four different pathways, 1) monooxygenation of the aromatic ring to form dimethylcatechols followed by extradiol ring-cleavage as reported for 2,3- and 3,4-DMP degradation [28, 31], 2) oxidation of the methylsubstituent to form methyl-substituted gentisates followed by degradation through a gentisate degradative pathway as reported for 2,5-, and 3,5-DMP degradation [32, 33]; 3) oxidation of both methylsubstituents to form 4-hydroxyisophthalate followed by hydroxylation and degradation via a protocatechuate pathway as reported for 2,4-DMP degradation [34, 35] or 4) two successive monooxygenation of the aromatic ring 2,6-dimethylhydroquinone and 2,6-dimethyl-3-hydroxyhydroquinone as reported for 2,6-DMP degradation [11]. Proteome analysis showed the expression of a catechol *meta* cleavage pathway by *Delftia* sp. LCW in the presence of 3,4-DMP and 2,3-DMP including a catechol 2,3-dioxygenase, a 2-hydroxymuconic semialdehyde dehydrogenase and a 4-oxalocronate tautomerase. Catechols will be formed by a phenol monooxygenase, where the gene encoding the alpha-subunit (P3 subunit) was also detected in the genome through PCR, in

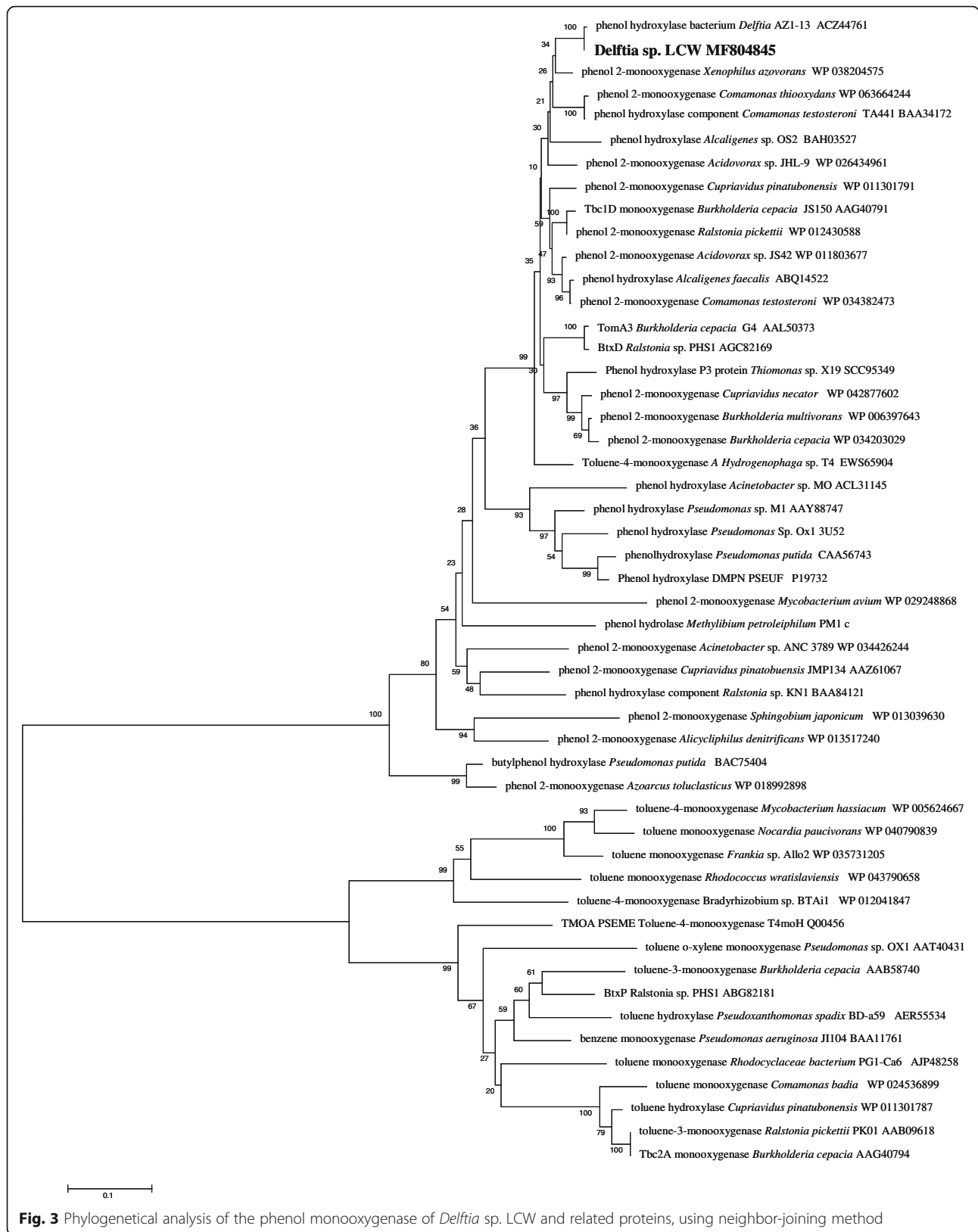


Fig. 3 Phylogenetical analysis of the phenol monooxygenase of *Delftia* sp. LCW and related proteins, using neighbor-joining method

Table 2 Identified proteins of *Delftia* sp. involved in phenol catabolic pathways

Treatment	Entry	Protein names	Gene names	Organism	Peptide length (bp)	Inferred DMP -catabolic pathway
3,4-, 2,3- and mixtures	D1LCK1	Phenol hydroxylase large subunit		Bacterium AZ1-13	186	Catechol <i>meta</i> and <i>ortho</i> cleavage
3,4- 2,3- and mixtures	Q60GE8	Catechol 2,3-dioxygenase	ORF7NH	<i>Delftia acidovorans</i>	314	Catechol <i>meta</i> cleavage
2,3-	A9C0K2	Protocatechuate 4,5-dioxygenase	Daci_4445	<i>Delftia acidovorans</i> (strain DSM 14801 / SPH-1)	289	Protocatechuate <i>meta</i> cleavage
3,4-, 2,3- and mixtures	Q8KRR9	2-hydroxymuconic semialdehyde dehydrogenase	nahl	<i>Pseudomonas fluorescens</i>	486	Catechol <i>meta</i> cleavage
3,4-, 2,3- and mixtures	A0A1C7L505	4-oxalocrotonate tautomerase	ACM14_28930	<i>Delftia</i> sp. JD2	138	Catechol <i>meta</i> cleavage

order to produce the corresponding dimethylcatechol. A respective pathway for the degradation of 3,4-DMP was previously observed in *Pseudomonas* sp. CF600 and *Pseudomonas putida*. P35X [10, 31, 36].

Although catechol *meta* cleavage enzymes were detected in the presence of 2,3-DMP, an additional enzyme was identified when the isomer was provided as sole carbon source. This enzyme was the protocatechuate 4,5-dioxygenase (A9C0K2). Other proteins belonging to the protocatechuate

pathway were not detected in the proteomic analysis. The protocatechuate pathway has been described only in 2,4-DMP degradation by *Pseudomonas putida* NCBI MB 9866 where it is formed through the subsequent oxidations both methyl group of 2,4-xylene to form 4-hydroxyisophthalate and hydroxylation to protocatechuate [35]. Our results indicate that *Delftia* sp. LCW produced enzymes of the protocatechuate pathway in the presence of 2,3-DMP provided as a sole

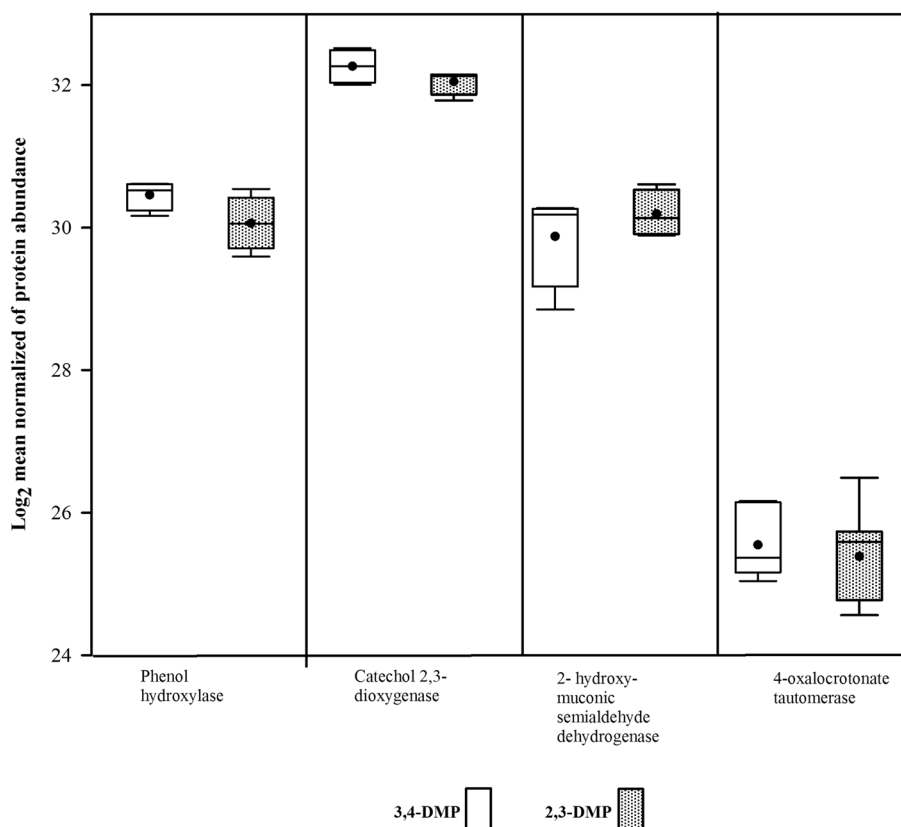


Fig. 4 Abundance of representative proteins involved in DMP degradation with 3,4- and 2,3-DMP as singles isomers. Bars indicate mean \pm SD. No statistical differences were found ($p > 0.05$)

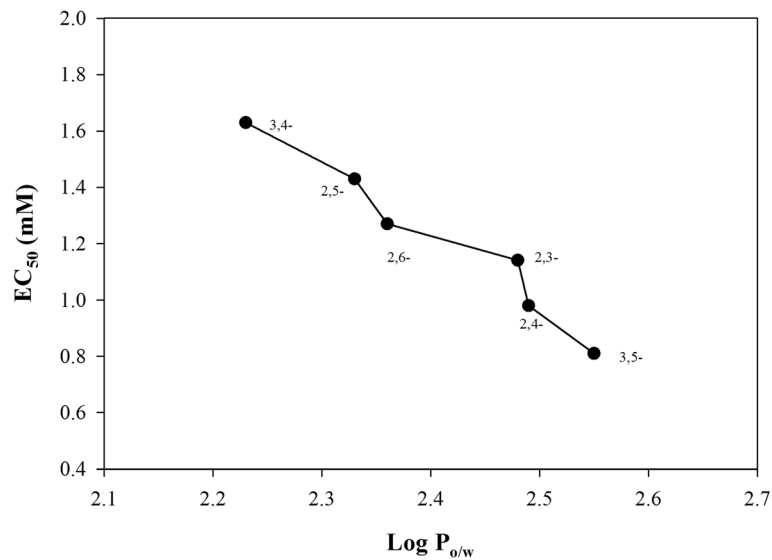


Fig. 5 Correlation between EC₅₀ (half maximal effective concentration) and Log $P_{o/w}$ (partition coefficient octanol/water) for the six DMP-isomers

carbon source. However, this is probably due to fortuitous induction. Since proteins were identified by searching against UNIPROT database for genera related to *Delftia* or reported DMP-degrading bacteria, only the anticipated proteins listed could be identified.

When a mixture of 3,4- and 2,3-DMP was provided, there was an evident shorter lag phase for the degradation of 2,3-DMP. Fostered degradation of DMP in mixtures with other aromatic compounds was previously observed in a sequencing batch reactor, where degradation kinetics of 3,4-DMP were improved in the presence of 4-nitrophenol (4-NP) [12]. Likewise, experiments with *C. testosteroni* had shown that the addition of phenol increased cell growth and shortened the time necessary for 4-Chlorophenol (CP) degradation [27]. Similar experiments with *Pseudomonas putida* XQ23 showed acceleration of 2,3-DMP degradation in the presence of DMP mixtures [37]. Those finding, in addition to higher abundance of *meta* pathway proteins in the mixture of the *ortho* DMP, suggested that the presence of 3,4-DMP in the mixture induced the expression of the catechol *meta* cleavage pathway and fostered the degradation 2,3-DMP.

Strain LCW was not able to grow on any of the other DMP isomers as a single substrate. Methylated phenols with methyl groups in ring position five (2,5 and 3,5-DMP), have been reported to be degraded by bacteria via gentisate pathway metabolism [32]. Proteins related to gentisate pathways were not detected in *Delftia* sp. LCW. Members of *Delftia* genus typically encode gentisate pathway. However, the appropriate methylhydroxylase has not been found in *Delftia* genomes, according to the search in PATRIC database. LCW strain transformed 2,4-, 2,5- and 3,5-DMP in the presence of

3,4-DMP. Mixtures of 3,4- and 2,3- DMP led to higher microbial yields than mixtures with 3,4- plus 3,5-, 2,5- or 2,4-DMP, showing the ability of *Delftia* sp. LCW to use only 3,4- and 2,3-DMP as a carbon source, and to remove 3,5-, 2,5- and 2,4-DMP by co-metabolic transformation. Similar co-metabolic transformation of 2,4-, 3,4- and 3,5-DMP has been reported for different strains of *Pseudomonas* [9] and *C. testosteroni* CPW301 [27]. It was also reported that *C. testosteroni* CPW301 was able to partially degrade 2,5- and 3,5- DMP by co-metabolism when growing with 4-CP and 4-MP [26]. In the case of *Delftia* sp. LCW, 3,4-DMP induced the enzymatic activity that allowed the transformation of 2,4-, 2,5- and 3,5-DMP and fostered 2,3-DMP degradation.

Screening of catabolic genes using degenerated primers and PCR led to the detection of the fragment of the phenol hydroxylase large subunit [38, 39]. The phenol hydroxylases comprise related family of enzymes capable to hydroxylate mainly phenols and their methyl-substituted derivatives to the corresponding catechols [40, 41]. Some phenol hydroxylases families have showed also the ability to transform toluene or benzene [42]. Phylogenetical relationships of phenol hydroxylase from LCW, showed that the closest protein is phenol hydroxylases from *Delftia* AZ1-13 (ACZ44761), which phenotype (L6) was a representative in a lab-scale reactor treating phenols [43]. Phylogenetical clustering of the phenol hydroxylase of LCW revealed similarities with the phenol hydroxylase *Comamonas testosteroni* TA441 (BAA34172) [44]. It was also similar to other strains with reported genes for catechol *meta* cleavage pathway e.g. *Cupriavidus pinatubonensis* [28, 45]. Another related phenol hydroxylase of LCW, belonging to

Pseudomonas sp. M1 (ABM96259) with high similarities to the *dmpKLMNOP* hydroxylase gen of CF600 [46].

In general, the catabolic pathway of 3,4- and 2,3- DMP by *Delftia* sp. LCW is similar to the one previously reported for *Cupriavidus pinatobuensis* JMP 134 [28], *Pseudomonas* sp. CF600 and *Pseudomonas putida* P35X [10, 29], *Pseudomonas aeruginosa* T1 [30] and *Comamonas testosteroni* JH5 [26]. Furthermore, phenol hydroxylases are reported to be of broad substrate specificity and to transform, phenol, 2-methylphenols, 2,4 and 3,4-DMP, *o*-cresol, *m*-cresol [28, 40, 47, 48], and are able to transform 2,4-DMP and 2,5-DMP [47]. Based on our findings, it is assumed that phenol hydroxylase of *Delftia* sp. LCW is not only responsible for the initial degradation of 2,3- and 3,4-DMP, but when induced by the presence of 3,4-DMP, it would be also able to transform other non-growth DMP isomers.

Delftia sp. LCW was also able to use benzene as growth substrate. One known pathway for the degradation of benzene involves two successive monooxygenations that are catalyzed by different soluble diiron monooxygenases [45]. Nevertheless, some phenol monooxygenases may catalyze both successive monooxygenations [40]. This characteristic was found in *Pseudomonas* sp. M1, where the multicomponent hydroxylase *phc* gene was responsible for the initial oxidation of phenol or benzene [46], this subunit is indeed similar to the one found in LCW. In addition, the phenol hydroxylase gene large subunit sequence found in LCW showed similarities with the toluene/benzene/chlorobenzene-monooxygenase (*tbc1D*) from *Burkholderia* sp. strain JS150 (AAG40791). Considering the detection of this gene sequence, the corresponding protein expressed and the phylogenetic similarities with other well-characterized functional proteins, we can propose that this phenol hydroxylase detected in *Delftia* sp. LCW may be responsible of catalyzing both monooxygenation reactions during the degradation of benzene.

Previous toxicity assays performed in bacterial strains such as *Vibrio fischeri*, showed highest toxicity of 3,4-DMP compared to other DMP-isomers, cresols, resorcinol and other dihydroxyphenols [4, 49, 50]. 3,4- and 2,3-DMP from activated sludge treating phenols-like wastewater had been reported to be toxic for eukaryotic multicellular organisms such as the ecotoxicological indicators *Daphnia magna* and *Thamnocephalus platyurus* [4]. In bacteria, there are cultures of *Pseudomonas* strains where the highest concentration of 3,4-DMP supporting growth was in the range of 2.5 mM [51], i.e. similar to the current findings with *Delftia* sp. LCW. The toxicity tests of the different DMP isomers on *Delftia* sp. LCW showed that 3,5-DMP was the most toxic isomer followed by a decreasingly toxic series in the following order: 2,4-, 2,3-, 2,6-, 2,5- and 3,4-DMP (Fig. 5).

Considering that only 3,4-DMP contributed to 8.5% for the phenol toxicity in ash heap water from an oil

shale industry [52] and given the fact that this isomer can impair the performance of biological treatment processes of phenolic wastewater [4], the tolerance and degradation of 3,4-DMP by *Delftia* sp. LCW, together with its role for co-metabolic transformation of other DMP isomers support the proposal of LCW strain as a promising microbial tool component on further developments of wastewater treatment technologies of phenolic compounds.

Conclusions

LCW strain was able to degraded complex aromatics compounds. DMPs and benzene are reported for the first time to be degraded by a member of *Delftia* genus. In this strain, the isomer 3,4-DMP acted as an inducer of phenol hydroxylase enzyme, that is responsible for a first oxidation of the DMPs, and it is followed by a further *meta* cleavage pathway for *ortho*-DMPs isomers. Such induction also led to the transformation of 2,4-, 2,5- and 3,5-DMPs. The higher resistance to DMP toxicity, and the understanding of degradation pathways for DMPs by LCW strain, as well as its origin as a rhizosphere bacterium from CWs, make of *Delftia* sp. LCW a suitable candidate to be used in bioremediation of coke-coal contaminated sites and is important to establish technological alternatives for wastewater treatment for coal-coke industry effluents.

Methods

Isolation and identification of DMP degrading bacteria

Bacterial strains were isolated by selective enrichment from a pilot-scale horizontal sub-surface CW fed with groundwater with benzene, phenols and *m*-cresols. In order to select strains with potential ability to degrade DMP for subsequent assays, bacterial growth on each of the six single isomers of DMP as a sole carbon source and energy was determined. The 16S rRNA gene from the only isolate showing growth on DMP isomers and called strain LCW, was amplified by PCR using universal bacterial primers 27F and 1492R [53]. Sanger sequencing of the purified PCR product was obtained (GATC Biotech AG, Cologne, Germany) and the 16S rRNA gene sequence was compared to public databases using RDP [54] to identify closest reference sequences. The 16S rRNA gene sequence from strain LCW is available in KY643688k with accession number KY643688 [55].

DMP degradation and co-metabolism assays

In order to evaluate degradation and co-metabolic capabilities of the isolated, pure cultures of the strain were transferred from plates to liquid LB medium and incubated overnight at 30 °C with agitation. Then, cells were harvested by centrifugation, washed three times with phosphate buffer (50 mM of NaH₂PO₄, pH 7.0) and re-suspended in 50 mL of liquid M9 [56] (turbidity of ~

0.05 at 620 nm) with the corresponding carbon source (as described below). First, the selected isolate was grown on 3,4-DMP and 2,3-DMP separately with a concentration of 70 mg L⁻¹ each. In addition, co-metabolic transformation by the strain was tested using each isomer (2,3-, 2,4-, 2,5-, 2,6- and 3,5-DMP) mixed with 3,4-DMP in concentrations of 35 mg L⁻¹ per isomer (i.e. 70 mg L⁻¹ DMP in total). Each treatment was set up in triplicates. Samples were taken from each culture every 4 and 12 h to determine cell number and DMP concentration, until the stationary phase was reached. Growth was followed by cell counting using a Coulter Counter® (Beckman Coulter Inc) and the software Multisizer 3 Version 3.51® (Beckman Coulter Inc.). The concentration of DMP in the cultures was measured in cell free supernatants by HPLC (Prominence, Shimadzu) equipped with a UV detector and a 2.7 µm by 3.0 × 150 mm poroshell 120 EC-C18 column (Agilent Technologies, USA). For the separation of the DMP isomers, the mobile phase A consisted of formic acid (0.1%) and phase B consisted of acetonitrile (100%), using the following gradients over a total run time of 45 min: 20% B to 40% B with a flow rate of 0.2 mL min⁻¹ [14]. For each treatment, a flask prepared under the same conditions without bacterial inoculum was set up in order to determine abiotic losses. In order to compare differences in degradation, the approximate bacterial yield for each mixture was calculated according to the following formula:

$$\text{Yield} = \frac{\text{Increase in bacterial biomass (mg dry biomass.mL}^{-1}\text{)}}{\text{C from DMP consumed (mg.mL}^{-1}\text{)}}$$

Increase in bacterial biomass =

$$\frac{(C_{tf} \text{ cell.mL}^{-1} - C_{t0} \text{ cell.mL}^{-1}) \times 3 \text{ mg. mL}^{-1*}}{1 \times 10^7 \text{ cell.mL}^{-1}} \text{ where,}$$

C_{tf} = number of cells per mL at the end of the exponential phase

C_{t0} = number of cells per mL at time zero

* A theoretical value of 3 mg mL⁻¹ of dry mass equivalent to 1 × 10⁷ cell mL⁻¹ for Gram negative rod bacteria was calculated [57]

$C \text{ from DMP consumed} = (DMP_{t0} \text{ mg. mL}^{-1} - DMP_{tf} \text{ mg. mL}^{-1}) \times 0.787^*$ where,

DMP_{t0} = Concentration of the corresponding DMP isomer at time zero

DMP_{tf} = Concentration of the DMP isomer at the end of the exponential phase

*1 M of DMP contains 78.7% of carbon

Growth of *Delftia* sp. LCW on other aromatic compounds

In order to investigate the spectrum of aromatics that *Delftia* sp. LCW was able to degrade, the strain was incubated with BTEX compounds. Initially, the strain was

grown on LB and incubated overnight at 30 °C with agitation. Cells were harvested by centrifugation, washed three times with phosphate buffer [50 mM of NaH₂PO₄, (pH 7.0)] and re-suspended in 50 mL of M9 medium (turbidity of 0.05 at 620 nm) and the selected sole carbon source at a concentration of 30 mg L⁻¹. The growth tests were performed in duplicate. In addition, flasks prepared under the same conditions without carbon sources were tested as a negative control. Aliquots were taken for turbidity measurements at 620 nm every 12 h for 2 weeks.

Detection and identification of aerobic catabolic genes in *Delftia* sp. LCW

Delftia sp. LCW was assessed for the presence of catabolic genes potentially involved in aerobic degradation of aromatic compounds. Detection of monooxygenases [38, 39, 58], intradiol catechol dioxygenases [59] and extradiol catechol dioxygenases [39, 60] in the genome of strain LCW was performed by PCR using previously described degenerate primers (Additional file 5). The obtained PCR products were purified and sequenced (GATC Biotech AG, Cologne, Germany). The obtained nucleic acid sequences were translated and compared to entries in public databases [61]. The only detected gene sequence from this PCR specific target survey is available in GeneBank with accession number MF804845. Protein translation was obtained through EMBL-EBI translation tool service [62], where, frame 3 from the positive strand was chosen. PATRIC genome database [63] was used in order to check the detected gene in other related genus members.

Proteomics analysis

Protein extraction, LC-MS measurements and protein identification were performed as described previously by Lünsmann et al., [64]. Briefly, for protein extraction, cells were grown on 3,4- DMP and 2,3-DMP individually and on mixtures of 3,4- with 2,3- and 3,4- with 3,5-DMP as described before. Bacterial cells were harvested in the middle of the exponential phase. Lysis was achieved by ultrasonication and proteins were recovered by denaturation and solubilization with urea buffer. The protein lysates were applied on a polyacrylamide gel electrophoresis. Gel pieces were cut and the proteins were lysed using trypsin overnight. The resulting peptide lysates were separated by liquid chromatography hyphenated with mass spectrophotometry (LC-MS). For the identification of proteins, the acquired LC-MS data were searched against the public database UniprotKB using the closest related genera, i.e. *Delftia*, *Pseudomonas*, *Ralstonia*, *Comamonas* and bacterium AZ1–13, as inferred from the sequenced Phenol hydroxylase fragment. LC-MS spectra were searched using the Proteome Discoverer (Thermo Fisher Scientific, v1.4, San Jose, CA, USA). Search settings were: Sequest HT search engine, trypsin

(full specific), MS tolerance 10 ppm, MS/MS tolerance 0.02 Da, two missed cleavage sides, dynamic modifications oxidation (Met), static modifications carbamidomethylation (Cys). Only peptides that passed the FDR thresholds of < 1% FDR q -value (Percolator) and rank 1 peptides were considered for further analysis. Label-free quantification was done using the Top-3 peptide area for approach. These linear area values were \log_{10} -transformed, median normalized and furthermore missing value imputation were performed. Principal component analysis (PCA) was performed using InfernoRDN (version 1.6044.35184 July 19, 2016). Hypothesis significance tests were performed with Prostar [65] using the threshold of the log fold change (FC) of 1 and the threshold of $-\log(p\text{-value})$ of 2 for protein abundances. The proteins significantly differing between incubations with the 3,4- and 2,3-DMP were functionally assigned with the annotation tool KEGG BlastKOALA [66].

Toxicity test

Delftia sp. LCW was grown in mineral medium M9 with 4 g L⁻¹ of disodium succinate as carbon and energy source in 50-mL flasks. Once the cultures reached the exponential phase of growth, DMP isomers were added separately at different concentrations (one flask per concentration) ranging from 45 to 350 mg L⁻¹. No DMP isomers were added to the control culture. For each flask, the OD_{620nm} was measured every hour until the growth stopped in one of the cultures flasks. To determine the toxicity of each compound, specific growth rate μ (h⁻¹), was calculated as follows [67]:

$$\mu [h^{-1}] = \frac{\ln x_{t_2} - \ln x_{t_1}}{t_2 - t_1} \quad \text{where,}$$

$$\mu [h^{-1}] = \text{Growth rate}$$

$$x_{t_1} = \text{OD}_{620} \text{ at time } t_1$$

$$x_{t_2} = \text{OD}_{620} \text{ at time } t_2$$

$$t_1 = \text{one hour after toxin was added [h]}$$

$$t_2 = \text{final time when the growth stopped [h]}$$

Inhibited growth (%) was defined as the percentages of the growth rates μ (h⁻¹) of toxified cultures divided by the growth rate of the control culture. EC₅₀ was calculated for each isomer by interpolation of the concentration resulting in 50% growth inhibition. Log P_{ow} values were obtained from Toxnet Database [68].

Statistical analyses

In order to indicate significant differences among values of maximal bacterial biomass in the cometabolic assay and protein abundances of selected proteins in proteomics data, normal distributions were tested with Shapiro Wilk and further parametric or not parametric test were performed accordingly. In addition, Pearson correlation was performed to correlate EC₅₀ and Log P_{ow} values for all isomers. Analyses were performed using SigmaPlot Version 13.0 (©Systat Software, Inc.). P -values and specific statistical tests are described in Additional file

Additional files

Additional file 1: Table A. p -values for the cometabolic assay, and **Table B.** p -values of paired t-tests for the comparison between 2,3- and 3,4-DMP. (DOCX 16 kb)

Additional file 2: Table S2. Proteome raw data for DMP treatments (XLSX 22980 kb)

Additional file 3: Figure S1. Principal component Analysis of the proteomic profile of strain LCW for the four DMP treatments. (PNG 37 kb)

Additional file 4: Figure S2. Proteins functional category of *Delftia* sp. LCW for the proteins with significant differences between 3,4- and 2,3-DMP isomers (proteins with p -value < 0.001 for FC were selected for the analysis). (TIF 100 kb)

Additional file 5: Table S1. Primers used for targeting catabolic genes in the genome of *Delftia* sp. LCW (DOCX 34 kb)

Abbreviations

BTEX: Benzene, toluene, ethylbenzene and xylenes; CL: Chlorophenol; COD: Chemical oxygen demand; CW(s): Constructed Wetland(s); DMP: Dimethylphenols; EC₅₀: Half maximal effective concentration; EMBL-EBI: European Molecular Biology Laboratory-The European Bioinformatics Institute; FC: Fold change; FDR: False discovery rate; HPLC: High performance liquid chromatography; KEGG: Kyoto encyclopedia of genes and genomes; KO: KEGG orthology; LB: Lysogeny broth; LC-MS: Liquid chromatography-mass spectrometry; LCW: *Delftia* sp. strain LCW; Log Pow: Partition coefficient octanol/water; MP: Methylphenol; NP: Nitrophenol; PATRIC: Pathosystems Resource Integration Center; PCA: Principal components analysis; RDP: The Ribosomal Database Project; UniprotKB: UniProt Knowledgebase

Acknowledgements

We thank Raúl Blázquez Arroyo, Marta Curiel Fernandez and Soeren Schmechta for the support during the isolation of the strain and preliminary tests. We thank Kathleen Eismann for the proteomics protocol performance. We thank the Helmholtz Centre for Environmental Research-UFZ - Department of Environmental Biotechnology and Department of Molecular Systems Biology for financial support and infrastructure.

Funding

Mónica A. Vásquez-Piñeros thanks COLCIENCIAS Departamento de Ciencia, Tecnología e Innovación- República de Colombia (Convocatoria 568-Doctorados en el exterior) for a sustaining grant for Doctoral studies. The funding institution had no additional role in the design of the study, collection, analysis, interpretation of data, nor in writing the manuscript.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

MAVP conceived the study, isolated the strain, performed the experiments, performed data analysis, and wrote the manuscript with significant input from all authors. PMML helped with the isolation strategy, NJ performed proteomic protocols and helped with the analysis of proteomic data. DHP helped with the phylogenetical and proteomic analysis and interpretation. CAR participated in the growth and degradation experiments. HH and HJ helped to conceive the study. HJH conceive the study, participated with toxicity tests and draft the manuscript. All authors contributed, read and approved the final manuscript.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

¹Helmholtz Centre for Environmental Research – UFZ, Department of Environmental Biotechnology, Permoserstr. 15, Leipzig, Germany. ²Technical University of Denmark, Research Data Management – DTU Library, Lyngby, Denmark. ³Helmholtz Centre for Environmental Research – UFZ, Department of Molecular Systems Biology, Leipzig, Germany. ⁴Helmholtz Centre for Infection Research – HZI, Microbial Interaction and Processes Research Group, Braunschweig, Germany. ⁵Helmholtz Centre for Environmental Research – UFZ, Department of Environmental Microbiology, Leipzig, Germany. ⁶Microbiomas Research Foundation, Bogotá, Colombia.

Received: 22 January 2018 Accepted: 30 August 2018

Published online: 06 September 2018

References

- Ahmaruzzaman M, Sharma DK. Adsorption of phenols from wastewater. *J Colloid Interface Sci.* 2005;287(1):14–24.
- Kahru A, Maloverjan A, Sillak H, Pöllumaa L. The toxicity and fate of phenolic pollutants in the contaminated soils associated with the oil-shale industry. *Environ Sci Pollut Res.* 2002;9(1):27–33.
- Autenrieth RL, Bonner JS, Akgerman A, Okaygun M, McCreary EM. Biodegradation of phenolic wastes. *J Hazard Mater.* 1991;28(1–2):29–53.
- Kahru A, Pöllumaa L, Reiman R, Rätsep A, Liiders M, Maloverjan A. The toxicity and biodegradability of eight main phenolic compounds characteristic to the oil-shale industry wastewaters: a test battery approach. *Environ Toxicol.* 2000;15(5):431–42.
- Zhou S, Watanabe H, Wei C, Wang D, Zhou J, Tatarazako N, et al. Reduction in toxicity of coking wastewater to aquatic organisms by vertical tubular biological reactor. *Ecotoxicol Environ Saf.* 2015;115:217–22.
- Veeresh GS, Kumar P, Mehrotra I. Treatment of phenol and cresols in upflow anaerobic sludge blanket (UASB) process: a review. *Water Res.* 2005;39(1):154–70.
- Vazquez I, Rodriguez J, Maranon E, Castrillon L, Fernandez Y. Study of the aerobic biodegradation of coke wastewater in a two and three-step activated sludge process. *J Hazard Mater.* 2006;137(3):1681–8.
- Hopper DJ, Kemp PD. Regulation of enzymes of the 3,5-xyleneol-degradative pathway in *Pseudomonas putida*: evidence for a plasmid. *J Bacteriol.* 1980;142(1):21–6.
- Viggor S, Heinaru E, Loponen J, Merimaa M, Tenno T, Heinaru A. Biodegradation of dimethylphenols by bacteria with different ring-cleavage pathways of phenolic compounds. *Environ Sci Pollut Res Int.* 2002;1:19–26.
- Ng LC, Shingler V, Sze CC, Poh CL. Cloning and sequences of the first eight genes of the chromosomally encoded (methyl) phenol degradation pathway from *Pseudomonas putida* P35X. *Gene.* 1994;151(1–2):29–36.
- Ewers J, Rubio MA, Knackmuss HJ, Freier-Schroder D. Bacterial metabolism of 2,6-xyleneol. *Appl Environ Microbiol.* 1989;55(11):2904–8.
- Tomei MC, Annesini MC. Biodegradation of phenolic mixtures in a sequencing batch reactor. *Environ Sci Pollut Res.* 2008;15(3):188–95.
- Jardinier N, Blake G, Mauchamp A, Merlin G. Design and performance of experimental constructed wetlands treating coke plant effluents. *Water Sci Technol.* 2001;44(11–12):485–91.
- Schultze-Nobre L, Wiessner A, Wang D, Bartsch C, Kappelmeyer U, Paschke H, et al. Removal of dimethylphenols from an artificial wastewater in a laboratory-scale wetland system planted with *Juncus effusus*. *Ecol Engin.* 2015;80:151–5.
- Vymazal J, Greenway M, Tonderski K, Brix H, Mander Ü. Constructed wetlands for wastewater treatment. In: Verhoeven JTA, Beltman B, Bobbink R, Whigham DF, editors. *Wetlands and natural resource management*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2006. p. 69–96.
- Faulwetter JL, Gagnon V, Sundberg C, Chazarenc F, Burr MD, Brisson J, et al. Microbial processes influencing performance of treatment wetlands: a review. *Ecol Engin.* 2009;35(6):987–1004.
- Wen A, Fegan M, Hayward C, Chakraborty S, Sly LI. Phylogenetic relationships among members of the *Comamonadaceae*, and description of *Delftia acidovorans* (den Dooren de Jong 1926 and Tamaoka et al. 1987) gen. nov., comb. nov. *Int J Syst Evol Microbiol.* 1999;49(Pt 2):567–76.
- Groenewegen PE, Breeuwer P, van Helvoort JM, Langenhoff AA, de Vries FP, de Bont JAM. Novel degradative pathway of 4-nitrobenzoate in *Comamonas acidovorans* NBA-10. *J Gen Microbiol.* 1992;138(Pt 8):1599–605.
- Hoffmann D, Müller RH, Kiesel B, Babel W. Isolation and characterization of an alkaliphilic bacterium capable of growing on 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid. *Acta Biotechnol.* 1996;16(2–3):121–31.
- Müller RH, Jorks S, Kleinsteuber S, Babel W. *Comamonas acidovorans* strain MC1: a new isolate capable of degrading the chiral herbicides dichloroprop and mecoprop and the herbicides 2,4-D and MCPA. *Microbiol Res.* 1999;154(3):241–6.
- Zhang C, Kang Q, Wang X, Zilles JL, Müller RH, Werth CJ. Effects of pore-scale heterogeneity and transverse mixing on bacterial growth in porous media. *Environ Sci Technol.* 2010;44(8):3085–92.
- González AJ, Gallego A, Gemini VL, Papalia M, Radice M, Gutkind G, et al. Degradation and detoxification of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) by an indigenous *Delftia* sp. strain in batch and continuous systems. *Int Biodeter Biodegr.* 2012;66(1):8–13.
- Yilmaz F, Içgen B. Characterization of SDS-degrading *Delftia acidovorans* and in situ monitoring of its temporal succession in SDS-contaminated surface waters. *Environ Sci Pollut Res Int.* 2014;21(12):7413–24.
- Vacca DJ, Bleam WF, Hickey WJ. Isolation of soil bacteria adapted to degrade humic acid-sorbed phenanthrene. *Appl Environ Microbiol.* 2005;71(7):3797–805.
- Rosch V, Denger K, Schleheck D, Smits TH, Cook AM. Different bacterial strategies to degrade taurocholate. *Arch Microbiol.* 2008;190(1):11–8.
- Hollender J, Dott W, Hopp J. Regulation of chloro- and methylphenol degradation in *Comamonas testosteroni* JH5. *Appl Environ Microbiol.* 1994;60(7):2330–8.
- Bae HS, Lee JM, Kim YB, Lee S-T. Biodegradation of the mixtures of 4-chlorophenol and phenol by *Comamonas testosteroni* CPW301. *Biodegradation.* 1996;7(6):463–9.
- Pieper DH, Stadler-Fritzsche K, Knackmuss H, Timmis KN. Formation of dimethylmuconolactones from dimethylphenols by *Alcaligenes eutrophus* JMP 134. *Appl Environ Microbiol.* 1995;61(6):2159–65.
- Bartilson M, Nordlund I, Shingler V. Location and organization of the dimethylphenol catabolic genes of *Pseudomonas* CF600. *Mol Gen Genet.* 1990;220(2):294–300.
- Ribbons DW. Specificity of monohydric phenol oxidations by meta cleavage pathways in *Pseudomonas aeruginosa* T1. *Arch Microbiol.* 1970;74(2):103–15.
- Shingler V, Franklin FC, Tsuda M, Holroyd D, Bagdasarian M. Molecular analysis of a plasmid-encoded phenol hydroxylase from *Pseudomonas* CF600. *J Gen Microbiol.* 1989;135(5):1083–92.
- Hopper DJ, Chapman PJ. Gentisic acid and its 3- and 4-methyl-substituted homologues as intermediates in the bacterial degradation of m-cresol, 3,5-xyleneol and 2,5-xyleneol. *Biochem J.* 1971;122(1):19–28.
- Pieper DH, Engesser KH, Knackmuss HJ. (+)-4-Carboxymethyl-2,4-dimethylbut-2-en-4-olide as dead-end metabolite of 2,4-dimethylphenoxyacetic acid or 2,4-dimethylphenol by *Alcaligenes eutrophus* JMP 134. *Arch Microbiol.* 1990;154(6):600–4.
- Chen YF, Chao H, Zhou NY. The catabolism of 2,4-xyleneol and p-cresol share the enzymes for the oxidation of para-methyl group in *Pseudomonas putida* NCIMB 9866. *Appl Microbiol Biotechnol.* 2014;98(3):1349–56.
- Chao H-J, Chen Y-F, Fang T, Xu Y, Huang WE, Zhou N-Y. HipH catalyzes the hydroxylation of 4-hydroxyisophthalate to protocatechuate in 2,4-xyleneol catabolism by *Pseudomonas putida* NCIMB 9866. *Appl Environ Microbiol.* 2016;82(2):724–31.
- Shingler V, Powlowski J, Marklund U. Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. *J Bacteriol.* 1992;174(3):711–24.
- Xiao Z, Huo F, Huang Y, Zhu X, Lu JR. A novel 2,3-xyleneol-utilizing *Pseudomonas* isolate capable of degrading multiple phenolic compounds. *Bioresour Technol.* 2012;104:59–64.
- Martinez-Lavancho PM, Chen Z, Lunsmann V, Marin-Cevada V, Vilchez-Vargas R, Pieper DH, et al. Microbial toluene removal in hypoxic model constructed wetlands occurs predominantly via the ring monooxygenation pathway. *Appl Environ Microbiol.* 2015;81(18):6241–52.
- Hendrickx B, Junca H, Vosahlova J, Lindner A, Ruegg I, Bucheli-Witschel M, et al. Alternative primer sets for PCR detection of genotypes involved in bacterial aerobic BTEX degradation: distribution of the genes in BTEX degrading isolates and in subsurface soils of a BTEX contaminated industrial site. *J Microbiol Methods.* 2006;64(2):250–65.

40. Pérez-Pantoja D, González B, Pieper DH. Aerobic degradation of aromatic hydrocarbons. In: Timmis KN, editor. Handbook of hydrocarbon and lipid microbiology. Berlin, Heidelberg: Springer Berlin Heidelberg; 2010. p. 799–837.
41. Leahy JG, Batchelor PJ, Morcomb SM. Evolution of the soluble diiron monooxygenases. *FEMS Microbiol Rev.* 2003;27(4):449–79.
42. Cafaro V, Izzo V, Scognamiglio R, Notomista E, Capasso P, Casbarra A, et al. Phenol hydroxylase and toluene/o-xylene monooxygenase from *Pseudomonas stutzeri* OX1: interplay between two enzymes. *Appl Environ Microbiol.* 2004;70(4):2211–9.
43. Basile LA, Erijman L. Maintenance of phenol hydroxylase genotypes at high diversity in bioreactors exposed to step increases in phenol loading. *FEMS Microbiol Ecol.* 2010;73(2):336–48.
44. Arai H, Akahira S, Ohishi T, Maeda M, Kudo T. Adaptation of *Comamonas testosteroni* TA441 to utilize phenol: organization and regulation of the genes involved in phenol degradation. *Microbiology.* 1998;144(Pt 10):2895–903.
45. Perez-Pantoja D, De la Iglesia R, Pieper DH, Gonzalez B. Metabolic reconstruction of aromatic compounds degradation from the genome of the amazing pollutant-degrading bacterium *Cupriavidus necator* JMP134. *FEMS Microbiol Rev.* 2008;32(5):736–94.
46. Santos PM, Sá-Correia I. Characterization of the unique organization and co-regulation of a gene cluster required for phenol and benzene catabolism in *Pseudomonas* sp. M1. *J Biotechnol.* 2007;131(4):371–8.
47. Arenghi FLG, Barbieri P, Bertoni G, de Lorenzo V. New insights into the activation of o-xylene biodegradation in *Pseudomonas stutzeri* OX1 by pathway substrates. *EMBO Rep.* 2001;2(5):409–14.
48. Jeong JJ. 3- and 4-alkylphenol degradation pathway in *Pseudomonas* sp. strain KL28: genetic organization of the lap gene cluster and substrate specificities of phenol hydroxylase and catechol 2,3-dioxygenase. *Microbiology.* 2003;149(11):3265–77.
49. Kahru A, Kurvet M, Külm I. Toxicity of phenolic wastewater to luminescent bacteria photobacterium phosphoreum and activated sludges. *Water Sci Technol.* 1996;33(6):139–46.
50. Kahru A, Pollumaa L, Reiman R, Ratsep A. Predicting the toxicity of oil-shale industry wastewater by its phenolic composition. *Altern Lab Anim.* 1999; 27(3):359–66.
51. Kahru A, Reiman R, Rätsep A. The efficiency of different phenol-degrading bacteria and activated sludges in detoxification of phenolic leachates. *Chemosphere.* 1998;37(2):301–18.
52. Kahru A, Kurvet M, Kurvet I. The study of toxicological impact of different compounds in complex mixtures: a case study on ash-heap water. *Rev Clin Pharmacol Pharmacokin.* 1997;11(2–3):137–41.
53. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol.* 1991;173(2):697–703.
54. RDP Naive Bayesian Classifier. [cited 10 November 2016]. Available from: http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp.
55. NCBI. National Center for Biotechnology Information Nucleotide. *Delftia* sp. strain LCW 16S ribosomal RNA gene, partial sequence. Available from: <https://www.ncbi.nlm.nih.gov/nuccore/KY643688>.
56. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory; 1989.
57. Neumann G, Cornelissen S, van Breukelen F, Hunger S, Lippold H, Loffhagen N, et al. Energetics and surface properties of *Pseudomonas putida* DOT-T1E in a two-phase fermentation system with 1-decanol as second phase. *Appl Environ Microbiol.* 2006;72(6):4232–8.
58. Baldwin BR, Nakatsu CH, Nies L. Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. *Appl Environ Microbiol.* 2003;69(6):3350–8.
59. Sei K, Asano K, Tateishi N, Mori K, Ike M, Fujita M. Design of PCR primers and gene probes for the general detection of bacterial populations capable of degrading aromatic compounds via catechol cleavage pathways. *J Biosci Bioeng.* 1999;88(5):542–50.
60. Brennerova MV, Josefiova J, Brenner V, Pieper DH, Junca H. Metagenomics reveals diversity and abundance of meta-cleavage pathways in microbial communities from soil highly contaminated with jet fuel under air-sparging bioremediation. *Environ Microbiol.* 2009;11(9):2216–27.
61. NCBI. National Center for Biotechnology Information. [cited 5 February 2017]. Available from: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>.
62. EMBL-EBI. [cited 12 April 2017]. Available from: <http://www.ebi.ac.uk/Tools/st/>.
63. PATRIC. Pathosystems Resource Integration Center. [cited 23 September 2017]. Available from: <https://www.patricbrc.org/>.
64. Lünsmann V, Kappelmeyer U, Taubert A, Nijenhuis I, von Bergen M, Heipieper HJ, et al. Aerobic toluene degraders in the rhizosphere of a constructed wetland model show diurnal polyhydroxyalkanoate metabolism. *Appl Environ Microbiol.* 2016;82(14):4126–32.
65. Wiczorek S, Combes F, Lazar C, Gai Gianetto Q, Gatto L, Dorffer A, et al. DAPAR & ProStaR: software to perform statistical analyses in quantitative discovery proteomics. *Bioinformatics.* 2017;33(1):135–6.
66. KEGG. Kyoto Encyclopedia of Genes and Genomes. KOALA Orthology And Links Annotation. [cited August 30 2017]. Available from: <http://www.kegg.jp/blastkoala/>.
67. Heipieper HJ, Löffeld B, Keweloh H, de Bont JAM. The *cis/trans* isomerisation of unsaturated fatty acids in *Pseudomonas putida* S12: an indicator for environmental stress due to organic compounds. *Chemosphere.* 1995;30(6):1041–51.
68. Toxnet. Toxicology Data Network. U.S. National Library of Medicine. [cited 2 December 2016]. Available from: <https://chem.sis.nlm.nih.gov/chemidplus/>.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

