



Sphingomonas herbicidovorans MH: a versatile phenoxyalkanoic acid herbicide degrader

HPE Kohler

EAWAG, Swiss Federal Institute for Environmental Sciences and Technology, Ueberlandstrasse 133, CH-8600 Dübendorf, Switzerland

Sphingomonas herbicidovorans MH was isolated from a dichlorprop-degrading soil column. It is able to grow on phenoxyalkanoic acid herbicides, such as mecoprop, dichlorprop, 2,4-D, MCPA, and 2,4-DB. Strain MH utilizes both enantiomers of the chiral herbicides mecoprop and dichlorprop as sole carbon and energy sources. Enantiomer-specific uptake systems are responsible for transporting the acidic substrates across the cell membrane. Catabolism is initiated by two enantiomer-specific α -ketoglutarate-dependent dioxygenases that catalyze the cleavage of the ether bond of the respective enantiomer to yield the corresponding phenol and pyruvate. Therefore selective degradation of the enantiomers of mecoprop and dichlorprop by strain MH is not only due to enantioselective catabolism but also to enantioselective transport.

Keywords: *Sphingomonas herbicidovorans* MH; phenoxyalkanoic acid herbicides; enantioselectivity; transport; α -ketoglutarate-dependent dioxygenase

Introduction

Sphingomonas herbicidovorans MH has been recently isolated and described [12,28]. Our studies of strain MH, which I summarize in this short review, are mainly driven by two reasons. First, strain MH degrades phenoxyalkanoic acid herbicides such as mecoprop, dichlorprop, 2,4-D, MCPA, and 2,4-DB (Figure 1, Table 1) [12,27,28]. The broad catabolic capabilities of strain MH with respect to the degradation of these herbicides, which are often found in leachates of landfills [5,10,13,29] and as groundwater contaminants [7], make this strain a potential candidate for bioremediation. Second, strain MH metabolizes the chiral phenoxypropanoic acid herbicides mecoprop and dichlorprop in an enantioselective manner. The effect of chirality on the metabolism of chiral pollutants, the importance of treating enantiomers separately, and the necessity of considering stereochemistry when studying degradation and environmental fate of chiral compounds have been emphasized in recent reviews [2,3,14,16]. Strain MH is an ideal system for studying the effect of chirality on the bacterial metabolism of chiral herbicides.

The chlorine-substituted phenoxyacetic acids 2,4-D, 2,4,5-T, and MCPA (Figure 1) were introduced as selective weed killers in the 1940s [18,26]. They are active against many broad-leaved weeds and are commonly used for the control of weeds in cereal crops, in grass pastures, and in lawns. The chiral 2-phenoxypropanoic acids mecoprop, dichlorprop, and fenoprop (Figure 1) were introduced in the 1950s for controlling certain weed species that were unaffected by the phenoxyacetic acid herbicides [18]. The herbicidal action of the phenoxyalkanoic acid herbicides is based on their ability to mimic natural plant growth regulators or

auxins [18]. These synthetic auxins induce several abnormalities in growth and plant structure; they cause dedifferentiation and initiation of cell division in certain mature cells and inhibit cell division in primary meristems, processes that ultimately lead to cell death. As early as 1953, studies on the stereospecificity of plant growth regulators revealed that the herbicidal activity of mecoprop and dichlorprop is associated only with the R enantiomer [19]. The phenoxyalkanoic acid herbicides are among the most widely used herbicides to control weeds in cereal crops throughout the world [26]. In the European Union (EU), the estimated total usage of 2,4-D, MCPA, mecoprop, and dichlorprop exceeded 11 000 tons in 1991 [7].

Strain MH belongs to the genus *Sphingomonas* [23–25], which appears to be ubiquitous in soil, water, and sediments. *Sphingomonas* strains isolated from these environments have broad catabolic capabilities that make them potentially useful for bioremediation and waste treatment [4]. The unique ability of *S. herbicidovorans* MH to cleave the ether bond of various phenoxyalkanoic acid herbicides makes it an interesting and fitting addition to this genus.

Taxonomic characteristics of strain MH

Sphingomonas herbicidovorans MH was isolated by Horvath et al [12]. They repeatedly cultivated a sample from a dichlorprop-degrading soil column (nine transfers) and used the sample to inoculate a chemostat. After 3 weeks of incubation in continuous culture, they isolated a dichlorprop-degrading strain from the effluent of the chemostat. Strain MH consists of Gram-negative, catalase- and oxidase-positive, yellow-pigmented, non-motile, and non-spore-forming rods [12]. It does not ferment carbohydrates, does not accumulate poly- β -hydroxybutyric acid under nitrogen-deficient conditions, does not use nitrate as an electron acceptor, and does not utilize complex carbohydrates [12]. Horvath et al [12] also noticed that strain

Correspondence: HPE Kohler, Department of Microbiology, EAWAG, Überlandstrasse 133, CH-8600 Dübendorf, Switzerland
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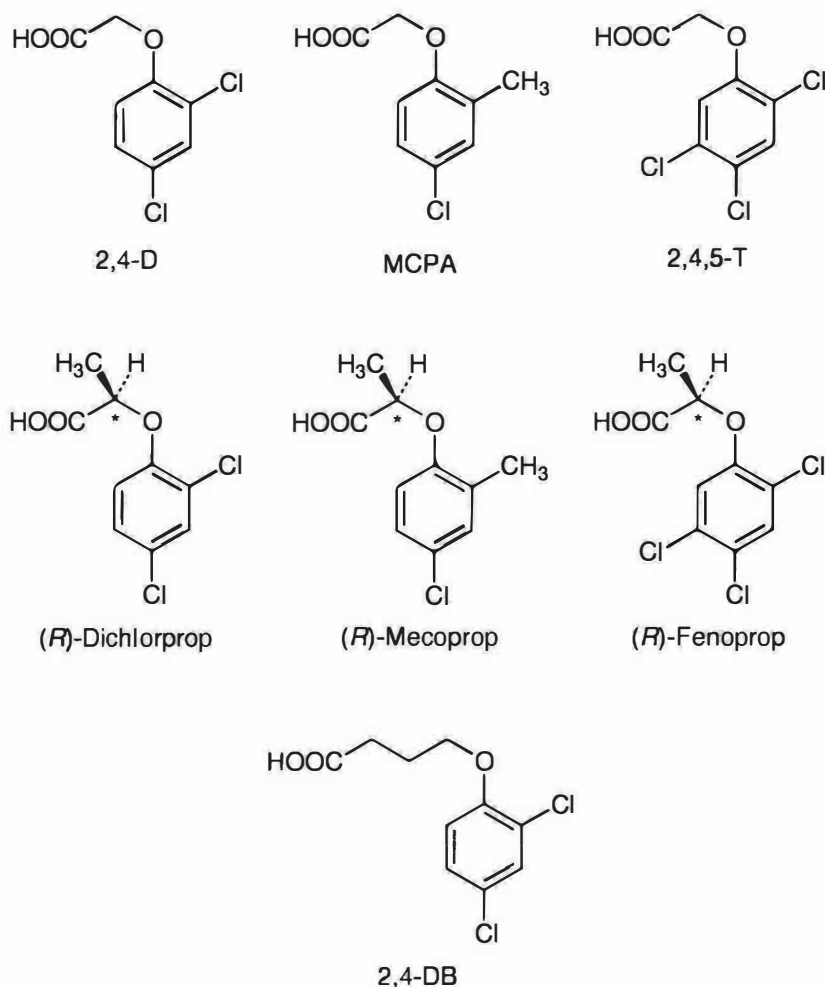


Figure 1 Structural formulas of the achiral phenoxyacetic acid herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), of the achiral phenoxybutanoic acid herbicide 4-(2,4-dichlorophenoxy)butanoic acid (2,4-DB), and of the chiral phenoxypropanoic acid herbicides (R)-2-(2,4-dichlorophenoxy)propanoic acid [(R)-dichlorprop], (R)-2-(4-chloro-2-methylphenoxy)propanoic acid [(R)-mecoprop], and (R)-2-(2,4,5-trichlorophenoxy)propanoic acid [(R)-fenoprop]. Asterisks mark the stereogenic centers.

MH releases a brownish water-soluble pigment into complex nutrient media. They tentatively identified strain MH as a *Flavobacterium* sp. Additional work on the taxonomic characteristics of strain MH by Zipper et al [28] and the Deutsche Stammsammlung von Mikroorganismen und Zellkulturen (DSMZ) [28] revealed that in addition to the characteristics mentioned above, strain MH is aminopeptidase-positive and alcohol dehydrogenase- and urease-negative, lyses only esculin but not gelatin or DNA, is not able to grow anaerobically, and does not form acids from sugars. Analysis of the cellular fatty acids showed that strain MH belongs to the *Sphingomonas* group within the α -subgroup of the proteobacteria [28]. The partial sequence of the gene for the 16S rRNA has the highest degree of similarity to the one for the 16S rRNA of *Rhizomonas suberifaciens* and *S. yanoikuyae*. DNA-DNA hybridization experiments between strain MH and the type strains of these two species showed that strain MH belongs to a new species within the group [28]. Although the highest identity was observed with *R. suberifaciens* (37.4%), the strain was named *S. herbicidovorans* because the genus name *Rhizomonas* is a

nomen rejiciendum (opinion 14) and, therefore, usage of this name is in violation of rule 28a of the International Code of Nomenclature of Bacteria [17]. The name *Sphingomonas herbicidovorans* MH was validated by inclusion on validation list No. 61 of the International Journal of Systematic Bacteriology [1].

Growth characteristics

Growth at 30°C on nutrient agar plates produces circular, bright-yellow colonies after 3 days; when the plates are incubated longer, the colonies change to a flower-like shape. Cells secrete a brownish pigment when growing on solid nutrient medium. In mineral salts medium with mecoprop as the sole carbon and energy source, strain MH requires supplements of vitamins and peptone for reproducible growth [28].

Table 1 lists growth substrates of *S. herbicidovorans* MH. Where available, growth rates, yields, and substrate consumption rates are included in the table. Strain MH is able to grow on the most important phenoxyalkanoic acid

Table 1 Growth characteristic of *S. herbicidovorans* MH

Growth substrate	Growth rate (h ⁻¹)	Substrate consumption rate (μM h ⁻¹)	Yield (g dry weight g ⁻¹ substrate)	Reference
(R)-Dichlorprop	0.082 ± 0.006	28	0.37 ± 0.06	[27]
(S)-Dichlorprop	0.036 ± 0.001	12	0.35 ± 0.02	[27]
Racemic mixture of dichlorprop ^a	NA ^b	NA	0.29 ± 0.03	[27]
-(R)-Dichlorprop ^a	0.043 ± 0.003	22	NA	[27]
-(S)-Dichlorprop ^a	0.029 ± 0.004	13	NA	[27]
Racemic mixture of dichlorprop	0.2 ^c	35	ND ^d	[12]
(R)-Mecoprop	0.032	14	0.30 ± 0.15	[28]
(S)-Mecoprop	0.039	13	0.35 ± 0.05	[28]
Racemic mixture of mecoprop ^e	0.016 ^f	NA	0.30 ± 0.04	[28]
-(R)-Mecoprop ^e	NA	6	NA	[28]
-(S)-Mecoprop ^e	NA	5	NA	[28]
2,4-D	0.133 ± 0.003	54	0.19 ± 0.05	[27]
MCPA	+ ^g	ND	ND	[28]
2,4-DB	+	ND	ND	[12]
2,4,5-T	- ^h	NA	NA	[12]

^aDiauxic growth; a growth rate was calculated for each enantiomer.

^bNA, not applicable.

^cThis growth rate is probably not correct. Recalculation of the data given in the reference showed that the growth rate must be between 0.065 and 0.115 per h.

^dND, not determined.

^eNo indication of diauxic growth; one growth rate was calculated.

^fIn this case the growth rate was affected by the concentration, which for each enantiomer was only half the value of what it was in the experiments with pure mecoprop enantiomers. Additional experiments showed increased growth rates in experiments with higher (RS)-mecoprop concentrations [28].

^g+, Strain MH grew on the compound.

^h-, Strain MH did not grow on the compound.

herbicides, such as mecoprop, dichlorprop, 2,4-D, MCPA, and 2,4-DB (Table 1, Figure 1).

S. herbicidovorans MH utilizes both enantiomers of mecoprop and dichlorprop as sole carbon and energy sources for growth [12,27,28]. However, growth experiments inoculated with cells that were grown on complex media and with the single pure enantiomers or the racemic mixtures as substrates revealed enantioselectivity; strain MH always degrades the S enantiomers before the R enantiomers. Growth on (S)-mecoprop is complete after about 3.5 days whether the R enantiomer is present or not. With racemic mecoprop as the growth substrate, degradation of the enantiomers is sequential, with (S)-mecoprop being degraded first [28]. However, the growth curve does not show indications of diauxic growth and, therefore, only one growth rate is reported in Table 1 for racemic mecoprop. (R)-Mecoprop is only slowly degraded during the first 50 h of such experiments, but its degradation rate markedly increases after the complete consumption of (S)-mecoprop. With pure (R)-mecoprop as the sole carbon and energy source, cells start to grow after a lag phase of about 7 days. Once growth commences, the cells grow as fast with (S)-mecoprop as the growth substrate (Table 1) [28]. Basically the same results were obtained with dichlorprop as the sole carbon and energy source [27]. As the growth substrate, (S)-dichlorprop completely disappears from the culture

medium after 43 h of incubation, while in experiments with (R)-dichlorprop as the growth substrate, a lag period of about 60 h is evident. When strain MH is offered racemic dichlorprop as the growth substrate, it degrades both enantiomers; the S enantiomer is preferentially degraded but at a slower rate (Table 1) [27]. In contrast to growth on racemic mecoprop, growth on racemic dichlorprop is diauxic and, therefore, two growth rates are reported in Table 1. Generally, chloride release is stoichiometric and strain MH does not excrete any detectable metabolites [12,27,28]. The levels of dissolved organic carbon at the beginning and at the end of a growth experiment usually correspond well to a complete conversion of dichlorprop and mecoprop [28].

Additional growth experiments showed that strain MH grows best on 2,4-D [27] (Table 1). Typically, growth on 2,4-D starts without a lag period and is complete after about 15 h of incubation.

Enantioselective metabolism of mecoprop and dichlorprop

Biochemical investigations focused on enzyme activities that turn over the mecoprop enantiomers [20]. *S. herbicidovorans* MH was grown on pure enantiomers and the harvested cells were disrupted. The crude cell extracts were tested for the existence of enzyme activities that were able to convert either (R)- or (S)-mecoprop. Cell extracts of *Sphingomonas herbicidovorans* MH grown on (R)-mecoprop contain an enzyme activity that selectively converts (R)-mecoprop to 4-chloro-2-methylphenol, whereas the enzyme activity in cell extracts of (S)-mecoprop-grown cells is selective for the S enantiomer. Both reactions depend on α-ketoglutarate and ferrous ions. Besides 4-chloro-2-methylphenol, pyruvate and succinate are formed as products of the reactions [20]. Labeling experiments with ¹⁸O₂ revealed that both enzyme activities catalyze a dioxygenation reaction. The appearance of ¹⁸O label in pyruvate and not in phenol suggests that the oxidative cleavage of the ether-bond proceeds through hydroxylation at the C-2 carbon of the propanoic acid side chain. The resulting unstable intermediate should then easily decompose to 4-chloro-2-methylphenol and pyruvate [20]. This suggestion was also in agreement with a general reaction mechanism for α-ketoglutarate-dependent dioxygenases. The reaction is thought to start with the formation of a ferryl oxidant (Fe_v=O) by the binding of oxygen to the iron cofactor, a process that is linked to oxidative decarboxylation of α-ketoglutarate to succinate [6,11,21,22]. Therefore, Nickel et al [20] proposed a degradation scheme based on two distinct α-ketoglutarate-dependent dioxygenases for the initial transformation of (R)- and (S)-mecoprop to 4-chloro-2-methylphenol and pyruvate by *S. herbicidovorans* MH (Figure 2) [20]. Both enzyme activities seem to share the overall chemistry with 2,4-D dioxygenase (TfdA), the enzyme catalyzing the first step of the degradation of 2,4-D by *R. eutropha* JMP134 [8,9], but not the substrate specificity. Whereas TfdA preferentially transforms 2,4-D to 2,4-dichlorophenol and glyoxylate (Figure 2) [8,9], the enzyme activities present in cell extracts of *S. herbicidovorans* MH specifically turn over either the R or the S enantiomer of mecoprop and show

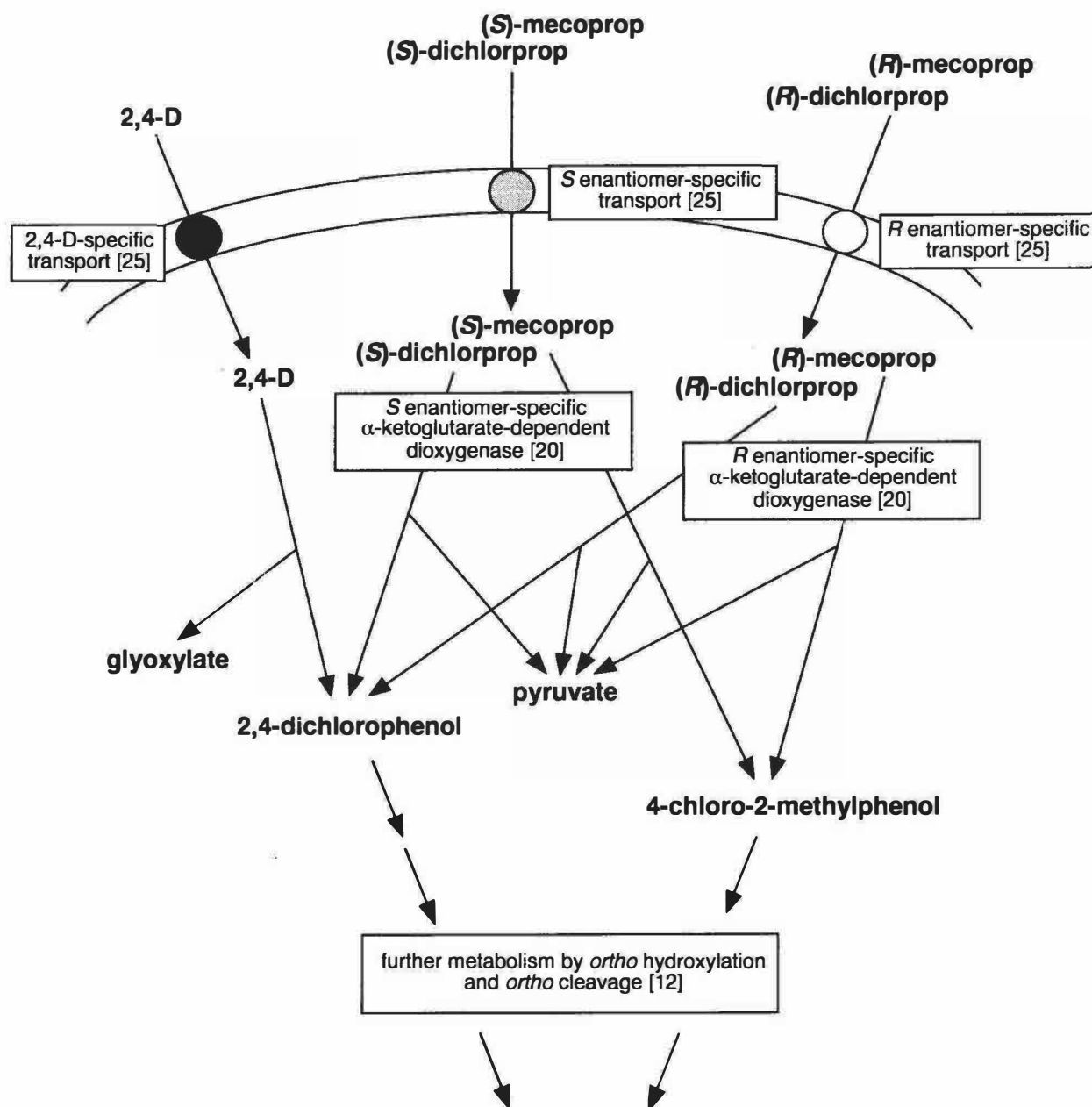


Figure 2 Model proposed for the uptake and metabolism of (R)- and (S)-dichlorprop, (R)- and (S)-mecoprop, and 2,4-D by *S. herbicidovorans* MH. Strain MH has three inducible proton gradient-driven uptake systems; one for (R)-dichlorprop and (R)-mecoprop, one for (S)-dichlorprop and (S)-mecoprop, and a third one for 2,4-D [27]. Once the compounds enter the cell, specific α -ketoglutarate-dependent dioxygenases enantioselectively catalyze cleavage of the ether bonds [20].

less activity with 2,4-D as the substrate [20]. (R)- and (S)-dichlorprop are also substrates of the (R)-enantiomer-specific enzyme and the (S)-enantiomer-specific enzyme, respectively; in these cases, 2,4-dichlorophenol and pyruvate were the common products (Figure 2) [15,20].

Enantioselective uptake of mecoprop and dichlorprop

Transport studies with ^{14}C -labeled dichlorprop enantiomers and with ^{14}C -labeled 2,4-D revealed that the uptake of (R)-dichlorprop, (S)-dichlorprop, and 2,4-D by *S. herbi-*

cidovorans MH is inducible [27]. The results from these transport studies clearly demonstrated that the uptake of dichlorprop is enantioselective and that there must be a separate uptake system for each enantiomer [27]. Additionally, Zipper et al [27] showed that the rates of uptake of 2,4-D by 2,4-D-grown cells in response to increasing 2,4-D concentrations are in accordance with saturation kinetics. The same is true for the transport of (R)-dichlorprop and the transport of (S)-dichlorprop into (R)-dichlorprop-grown and (S)-dichlorprop-grown cells, respectively. Experiments on the substrate specificity of the putative transport systems revealed that (R)-dichlorprop uptake is inhibited by (R)-

mecoprop but not by (S)-mecoprop, (S)-dichlorprop, or 2,4-D. On the other hand, (S)-dichlorprop transport is inhibited by (S)-mecoprop but not by (R)-dichlorprop, (R)-mecoprop, or 2,4-D [27]. From these results, Zipper et al [27] concluded that the (R)-dichlorprop carrier also transports (R)-mecoprop and that the (S)-dichlorprop carrier also transports (S)-mecoprop. Apparently, the transport systems are specific with respect to the substitution and the configuration at the stereogenic center (C-2 atom of the propanoic acid side chain) and rather unspecific with respect to the aromatic moiety of the phenoxypropanoic acids [27]. Moreover, transport of (R)-dichlorprop, (S)-dichlorprop, and 2,4-D is completely inhibited by various uncouplers and by the ionophore nigericin but is only marginally inhibited by the ionophore valinomycin and the ATPase inhibitor N,N'-dicyclohexylcarbodiimide [27]; these results are strong indications for the involvement of active transport. Zipper et al [27] concluded and summarized that the first step in the degradation of dichlorprop, mecoprop, and 2,4-D in *S. herbicidovorans* MH is active transport and that three inducible, proton-gradient driven uptake systems exist; one for (R)-dichlorprop and (R)-mecoprop, another for (S)-dichlorprop and (S)-mecoprop, and a third for 2,4-D (Figure 2).

Concluding remarks

The reaction sequence illustrated in Figure 2 demonstrates that selective degradation of the enantiomers of mecoprop and dichlorprop by the soil isolate *S. herbicidovorans* MH [28] is not only due to enantioselective catabolism [20] but also to enantioselective transport [27].

Taking into consideration the fact that the toxicity and the effect on biological targets might strongly differ for the enantiomers of a chiral pesticides or drug [16], study of enantioselective metabolism on the molecular level well deserves further emphasis.

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