# PESTICIDES REMOVAL USING PLANTS: PHYTODEGRADATION VERSUS PHYTOSTIMULATION

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## 1. Introduction

Pesticides are chemicals used for crop protection and pest control, and are probably the most widely distributed contaminants in the environment over the last century. Although it is extremely difficult to obtain precise figures concerning their production and use per country [1], millions of tons of pesticides are produced and spread annually all over the world. Thousands of different synthetic molecules are used as pesticides: carbamates, thiocarbamates, dipyridyls, triazines, phenoxyacetates, coumarins, nitrophenols, pyrazoles, pyrethroids, etc. Most of these chemicals contain chlorine, phosphorus, tin, mercury, arsenic or copper atoms. Pesticides are divided into different groups according to their target, e.g. herbicides, against weeds and toxic vegetation; insecticides, against harmful insects; fungicides, against blights, mildews, mould and rusts; algaecides, for the sanitary control of lakes, channels, water pools, reservoirs; bactericides, against some pathogenic microbes; etc.

To be efficient, a pesticide must remain in the appropriate environmental compartment and be stable enough to act against the target pest for a certain period of time. However, less than 5% of these products are estimated to reach the target organism, the remainder being deposited on the soil and nontarget organisms, as well as moving into the atmosphere and water [2].

Once in the environment, the persistence of a pesticide depends on its chemical stability, degradability by microorganisms, climatic conditions (influencing pesticide degradation through soil genesis), soil physicochemical properties (especially amount and nature of organic matter) and uptake by terrestrial and aquatic species including plants. The degree of environmental contamination is thus dependent on many factors and on physicochemical properties of the pesticide: volatility, reactivity, absorption and adsorption, solubility in water, partition between polar and non-polar phases (log Kow)



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and between soil and water (Kd). Depending upon their properties, many pesticides used in the field end up in surface and groundwater (Figure 1).

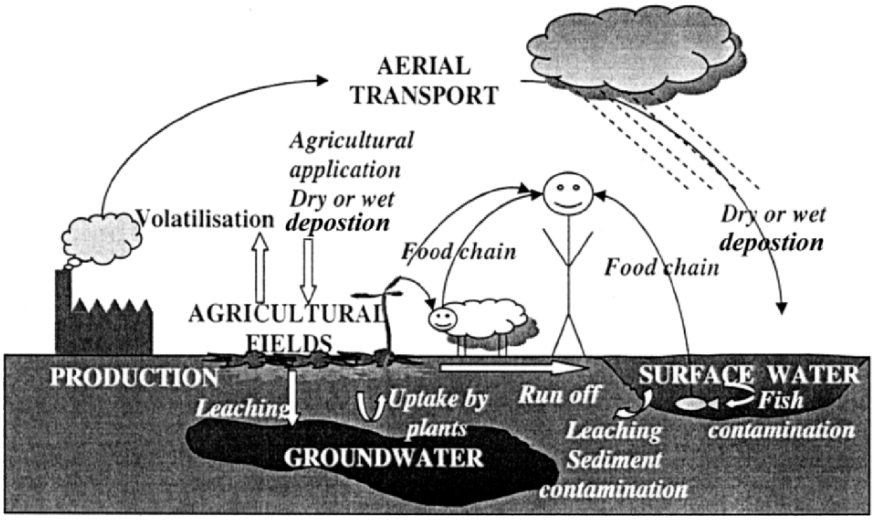


*Figure 1. Fate of pesticides in the environment.*

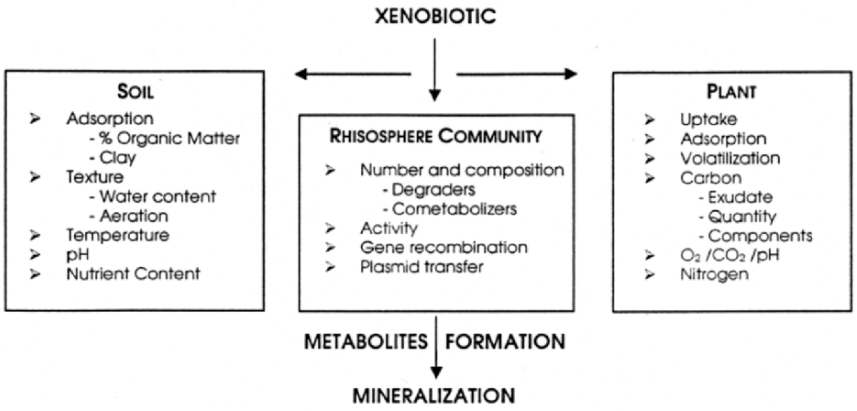


*Figure 2. Factors affecting the formation of metabolites and mineralization of pesticides in soil, plant and rhizosphere.*

Widespread and large-scale use of pesticides during the last decades has led to a global problem of pollution of soils and water resources. Heavy environmental contamination by pesticides may arise from industrial point sources such as accidental spillage during

production, wastewater from pesticide production plants, leakage from old stockpiles, storage and transport of pesticides, or as leachate from former dumping sites and municipal waste. The disposal of unwanted or obsolete pesticide stocks has also resulted in many long-term contaminated sites with very high levels of pesticides. In contrast, sources of pollution arising from agricultural use are considered to be diffuse as the compounds are distributed over large areas and at rather low concentrations [3].

Pesticides, like other organic pollutants, are more easily bioavailable in freshly spiked soils, as compared to aged or long-term contaminated soils [4-6]. The stability and toxicity of many pesticides make them hazardous when incorporated into the food chain. There is also increasing concern about their transformation products because these can be present at higher levels in soil than the parent pesticide itself. In some cases these products are even more toxic and more mobile, representing a greater risk to the environment than parent molecules.

A persistent organic pollutant does not undergo biodegradation in certain environments, whereas a recalcitrant compound resists biodegradation in most environments studied so far [7]. While partial biodegradation can be achieved by only one or a few biochemical reactions, total biodegradation involves more extensive metabolism leading to mineralization. Slow degradation rates often limit the practical use of microorganisms in remediating contaminated sites. Therefore, pesticides removal using plants and their interactions with rhizospheric microorganisms appears to be a promising approach, based on three basic principles (Figure 2):

- the accessibility or bioavailability of the contaminants to the biological system (plant roots, microorganisms);
- the biochemistry of plants or microbes, which transform the contaminant to a less toxic product; and
- the possibility of optimising the biological activity to efficiently remove the organic pollutant [8-12].

As examples of the potential of plants and rhizospheric microorganisms to remove pesticides from contaminated environment, we shall consider here only two pesticides with different properties: atrazine (ATR, 2-chloro-4-(ethyl-amino)-6-isopropylamino-*s*-triazine) and lindane (1, 2, 3, 4, 5, 6 hexachlorocyclohexane), amenable to different phytoremediation strategies, phytodegradation and phytostimulation, respectively.

## 2. Atrazine

Triazines are a large family of herbicides, widely used over the last decades, especially atrazine, simazine, propazine, prometryn and a few others (Figure 3). Even if the exact and present figures are not freely available, the annual use of atrazine (ATR) alone has been estimated to be around 40,000 tons in the USA [13], 5,000 tons in China [14] and more than 2,000 tons in Europe [15]. Triazines are pre-emergence, selective systemic herbicides used mainly for the control of annual grasses and broad-leaf weeds in a variety of cultivated crops, such as maize, sorghum, fruit orchards, sugar cane or cotton. Once taken up by the roots, triazines are evenly distributed throughout the plant via the xylem, and act in leaves by inhibiting photosynthesis.

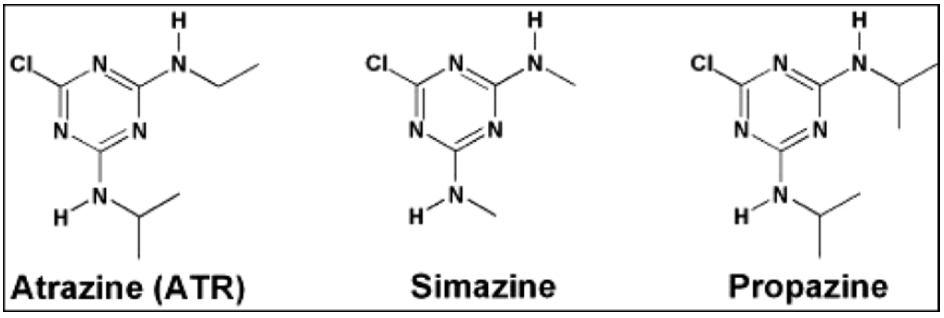


*Figure 3. Chemical structure of some triazines (log Kow between 2 and 3).*

To be effective, pre-emergence herbicides once applied to the soil, must have an agronomical remanence of several weeks or months, since they need time to exert their phytotoxicity and to kill any weeds germinating after their application. Herbicides are generally retained in the soil by adsorption in the superficial soil horizons, but washing can occur during the first rain or irrigation event following application. Additional losses occur through leaching to deeper soil horizons, and washing of soil particles from unvegetated surfaces. The potential danger of groundwater contamination has been recognized and it appears that atrazine and simazine application should be avoided in sandy soils, and should only be used in non-irrigated crops.

Leaching and agricultural runoff are therefore the primary mechanisms by which herbicides such as atrazine, and some of their degradation products, reach surface water and groundwater. Once atrazine reaches the surface water system, it is transported without substantial loss, and ends in lakes, reservoirs and alluvial aquifers, where it shows minimal loss by volatilization, sorption or transformation. Atrazine, other triazines and their metabolites are thus frequently detected in groundwater, lakes, streams and rivers of many countries [16].

Due to its persistence, atrazine is the second most common ground and surface water contaminant in the USA, and its metabolite deethylatrazine has also been found in groundwater and soils, sometimes at concentrations greater than that of the parent compound [17]. Moreover, atrazine is often used in combination with many other herbicides, such as alachlor, metolachlor, cyanazine, simazine, amitrole + simazine, or diuron + simazine. In most pesticide-contaminated agrochemical facilities, atrazine is thus found in combination with other widely used agricultural chemicals. Therefore, remediation strategies must often cope with a multiple-contaminant situation.

## 2.1. BIODEGRADATION, BIOREMEDIATION

In alkaline soils, a biological transformation of atrazine takes place, whereas in acidic soils both chemical and microbial transformations occur. By the action of bacteria, atrazine is mainly degraded to deethylatrazine (DEA, 6-Chloro-$N^4$-isopropyl-1,3,5-triazine-2,4-diamine) and deisopropylatrazine (DIA, 6-Chloro-$N^2$-ethyl-1,3,5-triazine-2,4-diamine) and to a lower extent to didealkylatrazine (DDA). In contrast, a chemical

dechlorination into hydroxyatrazine occurs in anaerobic environments and acidic soils. Dealkylates are thus the main metabolites of atrazine found in soils, and since they still have some herbicidal activity and are mobile compounds, they can also contaminate aquifers [17, 18].

Predominance of degradation by dealkylation has been shown by Shapir and Mandelbaum [19]: significant atrazine disappearance is observed in subsurface soil due to the activity of indigenous microorganisms in the upper part of soil, but only 1% mineralization occurs. Panshin *et al.* [20] observed that after atrazine application, DEA is the dominant degradation product detected the following year. It was shown that cultivated common soil microorganisms such as *Pseudomonas*, *Nocardia*, and *Rhodococcus* sp. are able to degrade atrazine, predominantly by dealkylation of the side chains on the triazine ring.

The limiting factor of atrazine degradation seems to be the absence of efficient atrazine-mineralizing microorganisms in soils, whereas atrazine-dealkylating bacteria are active. Although a few bacterial strains (such as the *Pseudomonas* sp. strain ADP) able to cleave the triazine ring and mineralize atrazine completely have been isolated and characterized, and their genetic potential estimated, the majority of bacteria in atrazine-contaminated fields cannot further metabolize the dealkylated products [21-25]. These bacteria initiate atrazine degradation with hydrolytic dechlorination, catalysed by ATZA (atrazine chlorohydrolase), followed by two amidohydrolytic reactions catalysed by ATZB and ATZC, which altogether transform atrazine to cyanuric acid, which can be mineralized to $CO_2$ and $NH_3$ by three other hydrolases, ATZD (cyanuric acid amidohydrolase), ATZE (biuret hydrolase) and ATZF (allophanate hydrolase). However, if *atzA*, *B* and *C* genes are widely distributed in different bacterial species, the other genes are less common. Other known genes involved in triazine degradation include *trzD* and *trzN*; *trzN* encodes the triazine chlorohydrolase, whereas *trzD* encodes the cyanuric acid amidohydrolase that catalyses the ring cleavage of cyanuric acid.

Although atrazine has been applied in the field for decades, its persistence in the environment and the absence of large scale demonstrations of microbial mineralization indicate the difficulty of rapid microbial breakdown in the field and the rarity of atrazine mineralizers. Many studies investigating the efficacy of bioremediation have been carried out on a bench scale and under ideal laboratory conditions. However, environmental conditions such as soil pH, temperature, nutrient availability and contaminant bioavailability vary from site to site and greatly affect the bioremediation process and growth of atrazine-mineralizers. Therefore, the removal of atrazine in contaminated soils by bioremediation is at present only a dream, and there is still a long way to go before a bioaugmentation approach can be successfully applied. Other biological tools should thus be explored, and phytoremediation appears to be the most promising [26-29].

## 2.2. PHYTOREMEDIATION

Phytoremediation or the use of vegetation at waste sites or contaminated soils can overcome limitations of microbial cleanup such as low bacterial population or inadequate microbial activity. Improving the quality of surface water and reducing

nonpoint source pollution can be achieved using wetland vegetation or vegetative filter strips to reduce herbicide runoff.

Plants can remediate environments contaminated with organic compounds directly via root uptake, detoxification by phytotransformation and conjugation with glutathione or sugars, and subsequent storage of nonphytotoxic metabolites in plant tissues; and/or indirectly by the release of exudates or enzymes which can enhance degradation by rhizosphere microorganisms [30, 31].

To be useful for the removal of herbicides from contaminated soil or preventing their runoff, a plant must first be able to grow in the presence of the target compounds without being harmed. The plant must not only be resistant to the pollutant, but also be able to remove it from the environment and to transform it into non-toxic metabolites or end-products. Differences in the ability of various plant species to accumulate and metabolize a particular pollutant have been shown, indicating that natural biodiversity should be better explored and exploited in order to choose the most appropriate plant species or variety in the development of any phytoremediation process [32]. Plant taxonomy and phytochemistry can help to use biochemical specificities of plants producing natural chemicals, whose structures are similar to xenobiotic compounds. Publications on plant metabolism of herbicides in species useful for phytoremediation are scarce compared to publications about plant metabolism for agronomic purposes. In the case of atrazine however, many results obtained in agronomy studies are useful when choosing the most appropriate families or genera for phytoremediation.

The use of vegetative filter strips is a low cost and practical option for improving the quality of runoff water from intensively farmed agricultural production areas [33]. Hybrid-poplar buffer strips were first initiated and planted in rows along a portion of a stream at the end of the 1980's [34]. Poplar is able to take up atrazine with transpiration stream, showing that such buffer strips are also effective in removing atrazine from agricultural percolation and runoff water. The only extensive study of plant metabolism of atrazine for a phytoremediation purpose is precisely in poplar (*Populus deltoides x nigra)*, able to transform the herbicide mainly into dealkylates and to a lesser extend, into polar ammeline [26, 27].

Grasses and semiaquatic plants can also remove nutrients and chemicals; reduce transport of contaminants like atrazine from runoff water by reducing flow which promotes deposition of sediment-adsorbed herbicides; and thus allow time for plant uptake and metabolism or infiltration of pollutants into soils and subsequent degradation before entering water systems. The use of common cattails (*Typha latifolia*) to remove simazine from contaminated water has also been sucessfully tested [31], whereas the atrazine mineralization potential of wetlands has been shown [35]; and a constructed wetland is able to treat efficiently atrazine present in nursery irrigation runoff [36].

Anderson and Coats [37] have evaluated the degradation of atrazine in rhizosphere soil of 15 plant species used for vegetative filter strips. Enhanced mineralization was found in rhizopshere soil collected from kochia (*Kochia acoparia*), common lambsquarters *(Chenopodium album*), foxtail barley (*Hordeum jubatum*), witchgrass (*Panicum capillare*), catnip (*Nepeta cataria*) and musk thistle (*Carduus nutans*). On the other hand, the efficiency of a natural filter of bluegrass (*Poa annua*) and fescue (*Festuca sp.*) strips located immediately down slope of a standard erosion plot of 9%

slope has been investigated [28]. Trapping efficiency of atrazine by a 4.5 m wide strip was 93%, in the same magnitude as dissolved phosphorus, nitrate, ammonium and sediments. This study emphasises the relevance of grass filters as buffer strips, but the mechanism underlying atrazine disappearance was not studied.

Decontamination of water polluted with 6 ppm atrazine by several marsh plants, common club-rush *(Schoeplectus lacustris)*, bulrush *(Typha latifolia)*, yellow iris (*Iris pseudacorus)* and common reed *(Phragmites australis)* was observed and the disappearance of atrazine was suggested to be due to the action of rhizosphere microorganisms [38]. The action of plants themselves was not explored, but was not excluded. Fernandez *et al*. [39] have also evaluated semiaquatic herbaceous perennial plants for their use in herbicide phytoremediation, such as canna *(Canna generalis)*, pickerel *(Pontaderia cordata*), and iris *(Iris x Charjoys Jan)*, and concluded that these taxa were not optimal for phytoremedation, since the plants exposed to herbicides showed significantly reduced biomass.

Phytoremediation can also prevent leaching of contaminants to groundwater from unplanted fields, after crop harvest [16, 31, 35]. On the other hand, higher rates of atrazine and simazine removal have been found in soil planted with *Pennisetum clandestinum* than in unplanted soil [29]. This could be due to plant uptake, degradation by enzymes secreted by plant roots, or increased microbial activity in the rhizosphere.

## 2.3. VETIVER AS A CANDIDATE FOR ATRAZINE PHYTOREMEDIATION

Vetiver (*Chrysopogon zizanioides* Nash) is a perennial tropical grass, native to India. Vetiver is by nature a hydrophyte, but often thrives under xerophytic conditions: it grows particularly well on river-banks and in rich marshy soil. It can withstand periods of flood, as well as extreme drought, survives temperatures of between –9°C and 45°C, is fire resistant, and is able to grow in any type of soil regardless of fertility, salinity, or pH. Vetiver is a tall (up to 3 meters high), fast growing perennial grass with stiff and tough stems which form a dense hedge with compact rhizome clumps (crown) when planted closely in rows [40].

The distribution of vetiver is pantropical, and some boundary strips are found in vetiver's native region of India. It was introduced recently in Southern regions of Europe, such as Italy, Portugal and Spain. Non-seeding vetiver plants are used in many countries for soil erosion control and many other applications: vetiver grass was first introduced for soil conservation and land stabilization in Fiji in the early 1950s. Recognizing the potential in combating land degradation, the World Bank has promoted the vetiver grass system since the mid 1980s, which is now used worldwide as a low-cost, low-technology and effective means of soil and water conservation and land stabilization in developing countries. The US Board of Science and Technology for International Development has reported successful vetiver applications for stabilization of slopes, terraces and channel banks in numerous tropical and subtropical countries. Vetiver plantation for soil erosion control is mainly performed linearly, along fields, terraces, canals, streams, or rivers, where the erosive force of water is at its greatest, lakeshores, artificial embankments, and little canals for irrigation or water drainage. It can even be planted across the river itself to slow down the flow of water [41].

As a result of the available literature it was deemed relevant to study vetiver uptake potential to intercept and remove not only atrazine, but also dealkylates from soil. The uptake of DEA and DIA was also tested because of their toxicity; relatively high bioavailability due to low sorption on soil matrix (log Kow 1.38 for DEA, and 1.7 for DIA); and their frequent occurence in groundwater, suggesting possible penetration and translocation in plants [42].

The ability of vetiver to take up ATR, DEA and DIA was thus investigated in a model system: 8-month old vetiver plants cultivated in hydroponics and under sterile and moderate transpiring conditions [43]. The disappearance of atrazine and dealkylates from the hydroponic system was dependent on transpiring flux of the plant, showing that the influence of microorganisms was negligible. This relationship was not linear because the concentration of the tested compounds was not constant: water was refilled to the initial level before each sampling. When the herbicide concentration was decreasing in the medium, a progressively lower quantity of herbicide was absorbed. After 20 days, uptake of DEA and DIA per liter of transpired water was not significantly different from that of ATR (Table 1).

*Table 1. Herbicide taken up by Vetiver plant per volume of transpired water. Values were calculated as cumulated quantity of herbicide taken up by the plant per total transpired water after 20 days exposure to 10 uM atrazine or dealkylates*

| Compound | Herbicide uptake per transpired water (mmol $l^{-1}$) |
|---|---|
| ATR | 5.20 ± 0.40 |
| DEA | 4.90 ± 0.05 |
| DIA | 4.80 ± 0.88 |

Vetiver could thus take up DEA and DIA in the same range as atrazine. This observation indicates that DEA and DIA uptake appeared to be largely a passive process closely associated with the movement of water, as reported by Wilson et al. [44] in *Canna hybrida*, by Raveton *et al.* [45] in *Zea mays* and *Acer platanus* protoplasts, and in *P. deltoides x nigra* by Burken and Schnoor [26, 27].

Since the uptake of DEA and DIA was passive and the transpiration stream constantly lowered their concentration in the roots, vetiver caused a global loss of dealkylates from the medium. The potential of vetiver for control of dealkylates produced by microorganisms in soil and atrazine runoff is believed to be high, since the plant was observed to absorb and tolerate atrazine, the first requirement of phytoremediation. The deep and dense root system of vetiver will physically retard the runoff of water loaded with atrazine and its primary metabolites, and retain soil and sediments on which atrazine and metabolites are adsorbed, allowing plant uptake and phytotransformation to occur, resulting in the removal of atrazine from the environment.

Since its leaf surface area is small compared to phreatophytes, and the highest uptake of atrazine and dealkylates is dependent on the volume of water transpired, vetiver is not thought to be very efficient for phytoremediation of highly contaminated soil or water. However, due to its highly dense root system, vetiver should be an ideal system against non-point pollution by atrazine and dealkylates. As vetiver is a huge grass, it is expected that this plant could remove pesticides in the same way as the smaller grasses, festuca

and poa, in temperate climates. Vetiver is not a crop plant, but has already been shown to have a wide range of applications; an understanding of the effect of vetiver on atrazine and dealkylates should open a new application window. In the near future, vetiver could play an important ecological role for water protection, especially in developing tropical countries. To our knowledge, most of the plants studied until now for phytoremediation of atrazine are adapted to temperate climates of developed countries.

The resistance mechanism of vetiver to atrazine was investigated to further the assessment of its potential for phytoremediation of atrazine-contaminated environments. Plants known to metabolise atrazine rely on hydroxylation mediated by benzoxazinones, conjugation catalyzed by glutathione-S-transferases and dealkylation probably mediated by cytochromes P450. All three possibilities were thus explored in mature vetiver grown in hydroponics. Whereas the role of benzoxazinones in the chemical hydroxylation of atrazine is only marginal [46], conjugation to glutathione was found to play a major role in the detoxification of atrazine by vetiver [47]. Dealkylates are further conjugated with glutathione in sorghum. Vetiver, close to sorghum, could also detoxify DEA and DIA by conjugation. This would be highly beneficial for the environment.

The use of a hydroponic system is the first step towards a comprehensive knowledge of the fate of pesticides in plants, but it is also a useful tool for the assessment of phytotreatments of industrial wastewater, agricultural runoff, surface and groundwater contaminated with pesticides. On the other hand, over-concentration of atrazine was observed in oil from roots grown in soil, suggesting that during plant ageing, partition might play a non-negligible role in retaining atrazine from agricultural runoff. Studies with other pesticides are required to see if vetiver as a tool against pesticide runoff could be extended to include other contaminants.

## 3. Lindane

The organochlorine 1, 2, 3, 4, 5, 6 hexachlorocyclohexane (HCH) is an efficient insecticide, available in two formulations: technical-grade HCH (a mixture of different isomers, mainly α, β, δ, and γ-HCH) and lindane (almost pure γ-HCH). The eight possible isomers differ in the axial or equatorial orientations of the different chlorine atoms (Figure 4). Typically, the technical mixture consists of 60-70% α-HCH, 5-12% β-HCH, 10-15% γ-HCH, 6-10% δ-HCH and smaller amounts of other isomers. Lindane, the only isomer with insecticidal properties, is isolated from technical-grade HCH by crystallization. It is a rather hydrophobic compound, with a log Kow of 3.72.

HCH is toxic and considered as a potential carcinogen, but because of its low-cost production and its effective pesticide properties, it is ubiquitously used in tropical countries to reduce vector-transmitted diseases, to protect livestock and to increase agricultural yields. Global use of lindane and technical HCH are estimated to be as high as 6 and 11 millions of tons, respectively. Its simple application, efficacy, and economic return may explain the popularity of this broad-spectrum insecticide, and why it was produced in large quantities worldwide until the discovery of its toxicity.

Being a persistent organic pollutant, technical-grade HCH can stay as long as 15 years after the last application in the field; HCH is nowadays found all over the world in

air, water and soil samples [1, 48-50]. In addition to agricultural soils, contaminated sites are found where isomers were disposed of in areas surrounding manufacturing centres. Remediation strategies are thus urgently needed to remove HCH isomers from environmental compartments so that they do not end up in food samples through growth of crop plants.

### 3.1. BIODEGRADATION, BIOREMEDIATION

The chemical structure and polarity of pesticides affect the solubility, sorption and volatility properties, and thus influence their transport, persistence and biodegradability. The ring structure of HCH prevents rotation around the C-C bonds, making vicinal elimination of chlorines dependent on the presence of axial chlorines oriented opposite to each other (anti-parallel positions). For example, β-HCH has no such chlorine pairs, since all chlorine atoms are equatorially oriented, which stabilize the molecule, and it is thus the most persistent isomer (Figure 4). In contrast, γ-HCH has 3 axial chlorines and 2 chlorine pairs, and α-HCH 4 axial chlorines and 1 chlorine pair, giving these isomers available sites for enzymatic attack (dehydrohalogenase). The physical properties and persistence in the environment of the different HCH isomers thus differ because of the different, axial or equatorial, chlorine orientations. Nevertheless, the four major isomers, having log Kow between 3.7 and 4.1, are considered as toxic and recalcitrant [50-52]. Even trickier, isomerisation of HCH can occur under both biotic and abiotic conditions. Bioisomerisation has been observed for bacterial cultures, as well as for bench-scale studies of sediment and soil slurries. This phenomenon should thus be taken into account in any bioremediation process, especially if the formation of the more stable β-HCH is significant.

HCH seems to be biodegradable under both oxic and anoxic conditions, but mineralization occurs only under oxic conditions. Biodegradation of HCH has been widely studied at laboratory scale, but information on full scale *in situ* bioremediation of industrial sites contaminated by HCH isomers, including lindane, is still very scarce [53]. Furthermore, the effect of HCH concentration on the biodegradation process is not yet known, especially in soils, but it has been recently reported that bacterial growth in liquid culture is decreased above 1 mM lindane and totally inhibited at 2.4 mM [54].

Lindane-degrading microorganisms have been isolated from different contaminated soils: among others, several *Clostridium* sp., *Pseudomonas* and especially *Sphingomonas paucimobilis* B90A (or *Sphingobium indicum*) and UT26 (or *Sphingobium japonicum*) [55]. Some of these strains are able to grow on γ-HCH as the only carbon source [52]. The aerobic degradation of lindane by *S. paucimobilis* is the best described and involves several novel enzymes encoded by *linA*, *linB*, *linC*, *linD*, *linE*, *linF*, and *linX* genes, leading to a possible mineralization. The LinA enzyme, a γ-HCH dehydrochlorinase, catalyses the dehydrochlorination of HCH to pentachloro-cyclohexene, then to 1,3,4,6-tetrachloro-1,4-cyclohexadiene. This metabolite is converted to 2,4,5-trichlorocyclohexenol, then to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) by the LinB protein, a halidohydrolase. LinC, as well as LinX, catalyse the oxidation

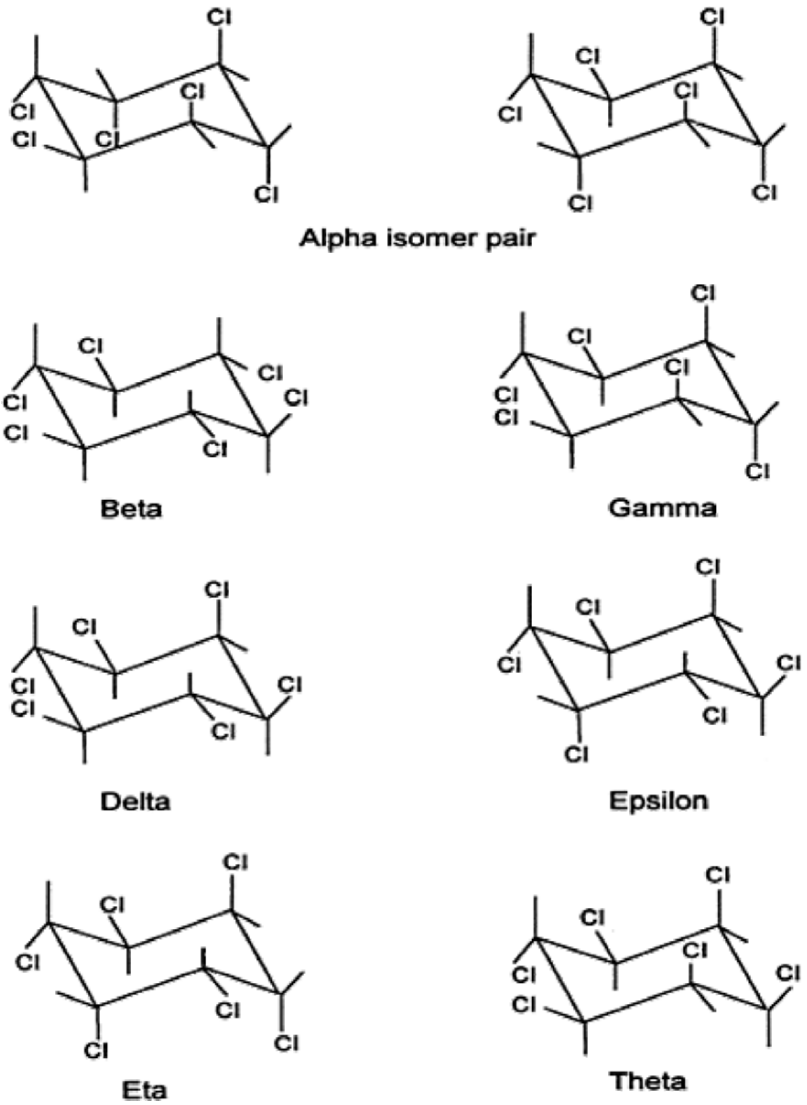


*Figure 4. Chemical structures of HCH isomers, showing the equatorial and axial positions of the chlorine atoms: Alpha = aaaaee; Beta = eeeeee; Gamma = aaaeee; Delta = aeeeee; Epsilon = aeeaee; Eta = aaeaee; Theta = aeaeee.*

of 2,5-DDOL to 2,5-dichlorohydroquinone (2,5-DCHQ) by a $NAD^+$-dependent dehydrogenase reaction. The *linD* gene encodes a reductive dehalogenase, catalysing the rapid conversion of 2,5-DCHQ to chlorohydroquinone, then very slowly to hydroquinone. The *linE* gene encodes a dioxygenase able to cleave the aromatic ring of

both chlorohydroquinone and hydroquinone, forming 2-chloromaleylacetate or maleylacetate (MA), respectively. MA is then reduced to β-ketoadipate by an MA reductase, the product of gene *linF* [56]. *LinA*, *linB* and *linC* are genes expressed constitutively, existing separately from one another on the genome, and belong to the so-called "upstream pathway". *LinD*, *linE* and *linF* are inducible genes forming the "downstream pathway"; *linD* and *linE* are organised within an operon with a transcriptional regulator called LinR, and are induced at 7 mg $l^{-1}$ γ-HCH, but not at 0.7 mg $l^{-1}$ [52, 57, 58].

*S. paucimobilis* is not the only aerobic HCH-degrading bacterium. *Escherichia coli* is also able to degrade lindane but only up to 10% level. Another bacterium, *Rhodanobacter lindaniclasticus,* degrades lindane with higher removal capacity [52]. Recently, two bacteria able to remove from 45.5% to 90% of lindane within 2 to 8 weeks of incubation were identified as *Pandorea* sp. [59]. These bacteria can use lindane as a sole source of carbon and energy. Some cyanobacteria, e.g. *Anabaena sp.* strain PCC7120, *Nostoc ellipsosporum* also possess lindane-degradation capacity [60]. It should be mentioned however that mineralization of lindane by such microorganisms is rarely encountered, with the exception of *S. paucimobilis* strains.

Several anaerobic bacteria, such as *Clostridium sphenoides*, *C. rectum*, *Citrobacter freundii* and *Desulfovibrio* sp. have been shown to degrade lindane by reductive dechlorination, but no mineralization seems to occur [52].

Finally, lignin-degrading fungi like *Phanerochaete chrysosporium*, *Trametes hirsutus*, *Cyathus bulleri* and several *Pleurotus* species have been shown to degrade lindane by extracellular lignin-degrading non-specific peroxidases [52].

Until now however, bioaugmentation with indigenous microorganisms failed to enhance degradation of either isomer [52]. Survival and activity of the inoculum are not always guaranteed, but the cultivation of appropriate plant species should increase the success of such a strategy. Bioremediation of HCH-contaminated soil is probably not realistic for crop fields where application rates were low, since HCH is expected to be removed by natural attenuation. In contrast, bioremediation should be appropriate and useful on industrial post-production or waste dumping sites, and storage sites.

### 3.2. PHYTOREMEDIATION

The uptake of pesticides by plants depends on the physicochemical properties of the compound, mode of application, soil type, climatic factors and plant species. Once in the roots, the chemical may be translocated to shoot via xylem. The permeation from plant roots to xylem is optimal for moderately hydrophobic molecules, with a log Kow between 0.5 and 3.5. More hydrophobic chemicals tend to bind with lipid membranes or oil possibly present in plant roots. Translocation of non-ionic pesticides thus varies greatly between plant species and depends on the properties of the chemical. Therefore, the uptake and translocation of hydrophobic compounds (log Kow > 4) is limited, and consequently so is their phytodegradation. On the other hand, the transformation of pesticides by rhizosphere microorganisms could result in metabolites more efficiently absorbed and translocated by plants. Thus any factor enhancing rhizospheric microbial activity should also increase the overall efficiency of pesticide phytoremediation.

With regards to HCH isomers, phytoextraction should not be favoured, since they are hydrophobic chemicals, theoretically bound to soil particles and also to plant roots, thus preventing plant uptake [61-64]. However, it has been recently shown that the predicted partition of lindane with lipids using the log Kow is notably lower than the measured sorption in roots and shoots of ryegrass and wheat seedlings, due to underestimation of the plant lipid contents and to the fact that octanol is less effective than plant lipids as a partition medium [65].

Nevertheless, the persistence of all isomers was found to be lower in cropped plots of maize (*Zea mays*), wheat (*Triticum* sp.) or pigeon pea (*Cajananus cajan*) than in uncropped plots, therefore sustaining the idea of remediation of polluted sites with plants [66]. Revision of available literature concerning contamination of the aerial part of plants reveals that the α, β and γ isomers have been detected in many plants, including *Lactuca sativa* (lettuce), *Sesamum indicum* (sesame), *Hydrilla verticillata* (hydra), *Lagernia siceraira* (bottle gourd), *Memordica charantia* (bitter gourd), *Luffa cylindrical* (sponge gourd), *Citrullus varifistulosus* (tinda punjab), *Spinacia oleracea* (spinach) and *Brassica campestris* (rape). These species were not selected for testing in hydroponics however, since the detected residues were due to a direct contact with lindane, and not as a result of translocation from roots to shoots. Barriada-Pereira *et al.* [67] also attributed the presence of lindane in the shoots of *Rubus ulmifolius* and *Paranthropophytia* to atmospheric deposition and not translocation. However, chilli (*Capsicum annuum*) and coriander (*Coriander sativum*) cultivated in lindane-contaminated soil were reported to contain γ-HCH residues in their aerial parts [43]. This fact was considered as a clue for the selection of plants able to take up lindane from a hydroponic experimental system. Within 9 days, lindane concentration in the medium decreased by 70% with chilli and 86% with coriander (Figure 5). Adsorption on roots of chilli and coriander accounted for 29% and 40% of γ-HCH disappearance, respectively. The 23% and 30% remaining loss was called "plant effect", which could include the increasing of pH from 5.0 to 6.8, leading to enhanced hydrolysis, and a possible uptake of γ-HCH in unknown quantities with the transpiration flux [43, 68]. However, the translocation of lindane from roots to shoots should be low, due to lindane hydrophobicity, unless special molecules are produced by plants, able to increase the apparent aqueous solubility of hydrophobic pollutants, such as those shown by Campanella and Paul [69] for dioxin absorption by zucchini (*Cucurbita pepo* L.).

In further experiments, vetiver plants were grown in Hoagland solution with $^{14}C$ lindane (2 mg/l). At the end of 30 days, it was observed that 12% of lindane disappeared from the solution. Since lindane was found to accumulate in vetiver roots where essential oil is produced, an attempt was made to use a plant, which has oil in the shoot as well. Lemon grass (*Cymbopogan citrates*) was thus grown in Hoagland solution for 19 days in the presence of $^{14}C$ lindane, but again more $^{14}C$ residues were found in roots (11.4%) than in shoots (4.2%). All the plants tested for uptake of $^{14}C$ lindane, *Iris* sp., *Sesuvium portulacastrum*, *Zea mays* and *Cymbopogan* sp. showed that $^{14}C$ residues were retained in roots and there was no significant translocation to shoots.

Another study has shown that the concentration of lindane in ryegrass, cultivated in hydroponics, slowly increases with uptake time to reach a plateau after a few days, indicating that plant metabolism and formation of bound residues are minimal in such a

system [70]. Other authors [71, 72] aimed to evaluate the bioaccumulation of HCH isomers in plants growing on areas surrounding a production centre and to investigate

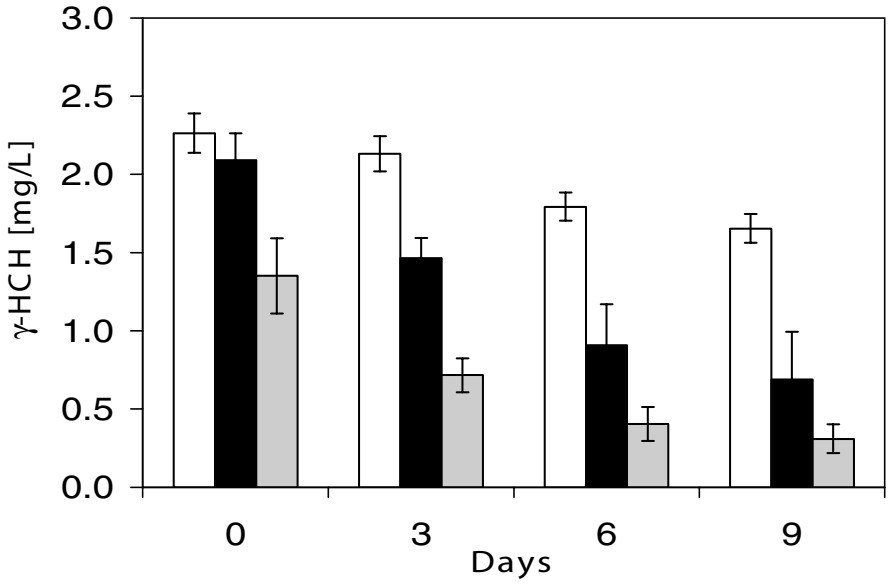


*Figure 5. Time course experiment of lindane disappearance in solution with chilli and coriander. Controls (white columns), chilli (black columns), coriander (grey columns). Data points represent the means of 5 replicates determinations. Bar = SD.*

the effect of the rhizosphere on these compounds. Five plant species were harvested and separated into roots, stems and leaves: *Chenopodium vulgare*, *Solanum nigrum*, *Cytisus striatus*, *Vicia sativa* and *Avena sativa*. Concentrations of total HCH in plant organs ranged between 1.7 and 62.5 mg $kg^{-1}$, depending on plant species and organs. Leaves systematically contained the highest amount of HCH, probably due to the volatilization of HCH isomers from the soil surface and subsequent sorption by leaves. In one species, C. striatus, the metabolites pentachlorocyclohexene, cyclopentiltrichloroethene and 6 trichloro, 4-en-hexanoic derivates were also detected in the all plant tissues analysed [71]. Data obtained from the bulk and rhizosphere soils from *C. striatus* and *A. sativa* suggest that both plant species tend to reduce the levels of all HCH isomers in the rhizosphere. This could be due to the enhanced biodegradation in the rhizosphere, root exudation of enzymes able to dechlorinate HCH isomers and/or sequestration by partitioning into the lipophilic plant tissues or uptake by the roots.

In the case of HCH, phytoremediation has thus shown its own limitations, even in the favourable case of hydroponic systems, where the pollutant is made highly bioavailable. Since rhizosphere microbial activity is known to aid the release of bound pesticide residues in soil, which can in turn enhance uptake and transformation, by plants, a

combination of bioremediation and phytoremediation, or the phytostimulation of rhizosphere microorganisms, is likely to be more successful.

Phytostimulation of bacteria present or added in soil seems the most promising approach to remove lindane from contaminated sites. It has long been known that plants release a vast range of organic materials through roots into the rhizosphere. These exudates contain water soluble, insoluble and volatile compounds including sugars, amino acids, organic acids, flavonones, phenolic compounds and even enzymes [12, 73-77]. The root exudates can enhance the acquisition of nutrients by plants; stimulate microbial growth in the rhizosphere; and change pH, water flux and availability of oxygen. Microorganisms able to use phenolic compounds as a carbon source often have enzymes that can co-metabolise pollutants with similar structures. Plants have also a great capacity to release secondary metabolites having a surfactant activity, which is favourable for phytoremediation purposes. Thus the degradation of several chlorinated pesticides has been reported to be higher in a vegetated soil than a non-vegetated soil. For example, the biodegradation of HCH isomers is enhanced in rhizosphere soils of *Kochia* sp., as compared to bulk soil, even if the mechanism by which this occurs is not yet known [78].

A dynamic synergy does exist between plant roots and soil microorganisms. The microbial activity in the immediate vicinity of the root (rhizosphere) seems to offer a favourable environment for co-metabolism of soil-bound and recalcitrant chemicals. The microbial transformations of organic compounds are not always driven by energy needs, but also by the necessity to reduce toxicity for which microbes may have to suffer an energy deficit. Thus, the processes may be helped and driven by the abundant energy provided by root exudates. Certain soil microorganisms can also produce biosurfactant compounds that may facilitate the removal and degradation of organic chemicals by increasing their availability to plants. Plants can thus take advantage of an increased bioavailability of nutrients and degradation of phytotoxic soil contaminants.

A successful example of exploring rhizosphere microorganisms for the decontamination of pesticide-contaminated soils has been highlighted by the recent findings of a coordinated Indo-Swiss project [77]. The project was aimed at investigating possibilities to remediate agricultural soils contaminated with lindane, using a plant-rhizosphere system. Mineralisation of $^{14}C$ lindane by rhizospheric soils of plants growing in lindane-contaminated fields indicated that microorganisms capable of degrading the insecticide were present. Enrichment culture techniques resulted in the isolation of bacteria growing in the presence of lindane. *Klebsiella* sp*., Pseudomonas* sp*.,* and *Pseudoarthrobacter* sp. degraded up to 50% of lindane with the formation of 2,3,4-trichlorobenzene. Root exudates of some plants stimulated the growth of lindane degrading bacterium *Pseudomonas* sp., indicating the need for an approach, which includes both plants and interacting microorganisms for an efficient degradation of pesticides. To treat lindane-contaminated soils, it thus appears that phytostimulation would be the most appropriate technique.

Soil composition influences sorption, soil pH, bulk density and water retention, all of which affecting aeration, nutrient availability and thus bioavailability and biodegradability of contaminants [61]. A high density of indigenous *S. paucimobilis* was found in the plant debris fraction of soil and it was postulated that plant organic matter

integrated into the soil aggregates served as a microhabitat rich in growth substrates. This can be the basis for the application of plant-derived organic amendments to soil as a phytostimulation strategy, like rice straw or other cellulosic material, because of their efficiency, availability in large quantities and low cost [52]. For example, the Daramend® technology for bioremediation of HCH-contaminated soils is based on the application of solid plant-derived organic matter providing nutrients and a non-toxic habitat for indigenous microorganisms; it creates a concentration gradient that facilitates diffusion of organic contaminants from pockets of higher to lower concentrations on the amendment surface, where they are more bioavailable [79]. Since the texture, nutrient requirements and microbial populations of each soil are specific, the usefulness and composition of amendments should be assessed on a case-by-case basis.

Detailed strategies for optimising treatments on sites contaminated with HCHs remain to be established and could involve enhanced natural attenuation and optimisation of environmental conditions, to stimulate growth and biodegradation by indigenous microorganisms. Supplemental nutrients and/or organic amendments could be added to enrich the soil and stimulate the bacteria degrading HCH. Bioaugmentation with phytostimulation or a vegetative cover should also be tested, either by increasing the population of microorganisms able to degrade HCH isomers, or by increasing the bioavailability of the insecticide.

## 4. Conclusions and perspectives

Both examples developed here show that plants and soil microorganisms have certain limitations with respect to their individual abilities to remove and degrade organic pollutants like pesticides, and other molecules containing chlorines and/or aromatic ring structures. However, plants and bacteria have very specific and complementary metabolic pathways, and their combined appropriate use can breakdown many man-made chemicals. Therefore, a synergy between rhizospheric microorganisms leading to increased availability of hydrophobic compounds and plants leading to their removal and/or degradation, may overcome many of the limitations, thus providing a sound basis for enhancing biological remediation of contaminated environments.

Phytostimulation or rhizoremediation is of particular importance because it refers to an important contribution that microorganisms in the root-zone (rhizosphere) make to the overall breakdown and removal of organic pollutants by plants. Plant-microbial interactions in the rhizosphere are thus of utmost importance for the degradation of recalcitrant chemicals in the environment [12, 30, 80].

However, further research into the mechanisms by which plants can stimulate biodegradation and the complexity of the soil-plant-microbe system due to its interwoven nature is thus required to better explore and exploit their huge potential. Such studies must be done not only at laboratory scale, but also under real conditions, as demonstration projects, to optimise the phytoremediation process and convince regulators and the general public of the technique's feasibility [81, 82]. To increase its acceptance as a remediation concept, phytoremediation must also become an economically interesting approach and biomass disposal or use after the treatment is thus an important issue to consider. For example, the biomass of fibres, oil or fragrance

producing plants like vetiver, could be used to recover these added-value products, if however their level of contamination is nil or low enough. Alternatively, contaminated biomass could be used for renewable energy generation, either by direct combustion, gasification or pyrolysis, or indirectly via biogas or biofuel production [83, 84].

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