related to the optical performances and to the energy at which the optical device is designed to work. In Table 1, the energy region from 500 eV to 50 keV is divided into three parts: low, medium and high energies.

At low energies, the material thickness needed to provide a π-phase shift becomes smaller (200–300 nm), but it is still necessary to achieve high aspect ratios of about 10 if one wishes to fabricate zones at the current lithographic spatial resolution limit[15,16]. With the latest generation of electron-beam machines and nano-structuring technologies[17,18], zone plates with a resolution of 20–50 nm have been fabricated for 200–500 eV X-rays, and alignment accuracy of ~10 nm has already been demonstrated[8]. Working at the lithographic limit, the outermost zone width of a quaternary FZP becomes twice as large as that of a binary FZP, thus doubling the optical resolution $\delta_{r,m}$. Therefore, at low energy, quaternary FZPs are complementary to binary FZPs, providing a good trade-off between the reduction of the optical resolution and the increase of the efficiency.

At medium energy, the typical phase-shifter thickness is of the order of one micrometre. Practical aspect ratios of 5–10, that can be achieved by today's very-large-scale integration (VLSI) technology, limit the minimum spatial resolution to 150 nm. At high energy, the phase-shifter thickness further increases to a few micrometres (see Table 1) and, accordingly, the minimum feature size reaches about 1 μm. In this range, where other optical devices are somewhat limited (although refractive lenses could improve in the future), quaternary FZPs show some unique advantages, especially with respect to their efficiency and optical resolution (see Table 1). Also, multilevel FZPs are compatible at high energy with microdiffraction, where the high focusing length matches the necessary low angular divergence.

With the consolidation of third-generation synchrotrons, multilevel FZPs could be used as focusing elements in research fields where high efficiency and high signal-to-noise ratio are needed at X-ray wavelengths. □

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Influence of environmental changes on degradation of chiral pollutants in soils

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Numerous anthropogenic chemicals of environmental concern—including some phenoxy acid herbicides, organophosphorus insecticides, polychlorinated biphenyls, phthalates, freon substitutes and some DDT derivatives—are chiral. Their potential biological effects, such as toxicity, mutagenicity, carcinogenicity, and endocrine disrupter activity, are generally enantiomer-selective, and different enantiomers are preferentially degraded (transformed) by micro-organisms in various environments[1–8]. Here we use field and laboratory experiments to demonstrate that environmental changes in soils can alter these preferences, and to suggest that the preferences shift owing to different groups of related microbial genotypes being activated by different environmental changes. In Brazilian soils, almost all pasture samples preferentially transformed the non-herbicidal enantiomer of dichlorprop ((*RS*)-2-(2,4-dichlorophenoxy)propionic acid), while most forest samples either transformed the herbicidal enantiomer more readily or as rapidly as the non-herbicidal enantiomer. Organic nutrient enrichments shifted enantioselectivity for methyl dichlorprop ((*RS*)-methyl 2-(2,4-dichlorophenoxy)propionic acid) strongly towards preferentially removing the non-herbicidal enantiomer in soils from Brazil and North America, potentially increasing phytotoxicity of its residues relative to that of the racemate. Assessments of the risks chemical pollutants pose to public health and the environment need to take into account the chiral selectivity of microbial transformation processes and their alteration by environmental changes, especially for pesticides as up to 25 per cent are chiral[9].

Enantiospecific biological effects associated with chiral chemicals are well recognized in the pharmaceutical industry where 50 of the top 100 most widely sold drugs, including barbiturates, ibuprofen, albuterol, and Ritalin, are marketed as single enantiomers to avoid adverse side effects[8,10]. The high cost of separating enantiomers

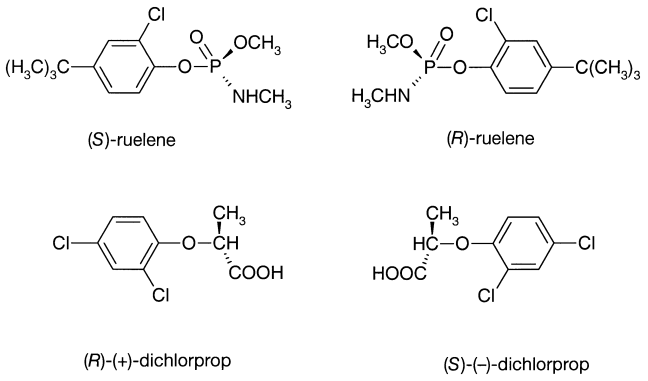

Figure 1 Enantiomers of ruelene and dichlorprop.

Table 1 Effect of deforestation on dichlorprop enantioselectivity in Brazilian soils

	n	$t_{1/2}$ (days)	c.v. (%)	(+) (%)	(–) (%)	(+/–) (%)
Methyl dichlorprop						
Forest	52	0.77	62	7.7	1.9	90
Pasture	75	0.63	49	11	1.7	87
Dichlorprop						
Forest	16	11	36	13	38	50
Pasture	37	16	57	6.7	93	0.0

Soil samples were collected from a forest in Rondonia, Brazil and from six deforested areas (pastures) nearby, ranging 9–47 years in age. Soil samples (1 gram) were amended with herbicides and incubated overnight for methyl dichlorprop and over ~6 months for dichlorprop. Selectivity for different enantiomers ((+) or (–)) was quantified as percentages of soil samples in which differences in the amounts transformed of each enantiomer were ≥15%. Non-selective samples (+/–) had concentration differences between (+) and (–) enantiomers of <15%. Transformation half-lives ($t_{1/2}$) of herbicide racemates were calculated ± a coefficient of variation (c.v.) among a number (*n*) of replicate soil samples. Almost all (93%) of the pasture samples preferentially transformed the non-herbicidal (–) enantiomer, while most forest samples either preferentially transformed the herbicidal enantiomer (13%) or transformed it as rapidly as the (–) enantiomer (50%).

Table 2 Effect of nutrient enrichment on enantioselectivity for methyl dichlorprop

	n	(+) (%)	(–) (%)	(+/–) (%)	Net preference (%)
Rondonia, Brazil					
Soil	127	9.0	2.0	88	7.0 (+)
Enrichment	340	15	50	35	35 (–)
Harvard Forest, Massachusetts, USA					
Soil	29	45	48	7.0	3.0 (–)
Enrichment	207	3.0	80	17	77 (–)
Risdalsheia, Norway					
Soil	16	0.0	88	13	88 (–)
Enrichment	80	15	74	11	59 (–)

Methods for testing soil were the same as Table 1, except that the soil samples contained beef extract and peptone. Net preferences, which indicated the extent to which soil (or soil + organic nutrients) preferentially transformed an enantiomer, were calculated as the absolute values of differences in percentage of soil samples preferring (+) or (–) enantiomers.

currently prohibits applying this technology to the commercial production of most pesticides and other industrial chemicals to reduce the unwanted effects of racemates as environmental pollutants. Notable exceptions include two herbicides, dichlorprop and mecoprop, in which a limited portion of the total worldwide production includes single enantiomers[11].

In the case of chiral pollutants, environmental studies have historically neglected to determine the adverse effects associated with particular enantiomers, and which enantiomers may persist in the environment. Consequently, much of the environmental data that has been collected for chiral pollutants, including many of the chemicals raising the greatest public concern, may have represented only the presence of relatively innocuous enantiomers.

To assess the persistence of different pollutant enantiomers across a wide latitudinal transect, and the possible influence of large-scale environmental changes on that persistence, we investigated enantioselectivity in soils collected from three areas after amending them with ruelene ((*RS*)-4-tert-butyl-2-chlorophenylmethyl-*N*-methyl phosphoramidate), dichlorprop, and methyl dichlorprop (Fig. 1). The sites included an upland plateau in Risdalsheia, Norway about 20 km inland of the North Sea, an 80-year-old mixed deciduous forest (Harvard Forest) in the northeastern US, and an area of the Fazenda Nova Vida about 250 km south of the city of Porto Velho in Rondonia, Brazil. Only the (+) enantiomers of dichlorprop and methyl dichlorprop are herbicidal[11] (see Methods). Both enantiomers of ruelene were insecticidal; however, the (+) enantiomer was four times more toxic.

Table 1 shows that tropical deforestation (conversion to pastures) had little or no effect on enantioselectivity or demethylation rates of the racemate of methyl dichlorprop. Demethylation was the first and most rapid step in the transformation process, which quantitatively produced dichlorprop as the reaction product. Soil warming in North America and Norway also had no measurable effect on racemate demethylation rates or enantioselectivity for methyl dichlorprop where field plots were heated 5 °C above ambient temperatures for ~7 years in North America and ~4 years in Norway[12,13]. Transformation of dichlorprop began after a 6-month lag period and proceeded 20 times more slowly (half-life of ~14 days compared with ~0.7 days for methyl dichlorprop).

Addition of inorganic fertilizers to forest and pasture plots in Brazil had no measurable effect on enantioselectivity for methyl dichlorprop (tested at 2 weeks and at 5 months after fertilization), or to plots in North America fertilized since May 1988. Laboratory enrichments with organic nutrients, which caused soils preferentially transforming the (+) enantiomer to shift to strongly preferentially transforming the inactive (–) enantiomer in samples from North America and Brazil (Table 2), did not have the same effect in samples from Norway where soils already preferentially removed the (–) enantiomer. There, microorganisms preferring the (+) enantiomer may have increased in activity somewhat after nutrient amendments, but most samples (74%) still preferred the (–) enantiomer. Preferential removal of (–) methyl dichlorprop would render residues more phytotoxic than the same concentrations of the racemate, as long as demethylation is a prerequisite step and the (+) dichlorprop enantiomer is not preferentially removed after nutrient enrichment.

Ruelene, like dichlorprop, was transformed by microbial populations only after about a 6-month lag period. The half-life, once transformation began, was ~40 days in Brazilian soils and 20 days in Norwegian soils. Ruelene racemate transformation rates were unaffected by deforestation or soil warming. Enantioselectivity, however, was affected in both cases. In Brazil, deforestation caused soils to shift from preferentially removing the less toxic ruelene (–) enantiomer (22% (+), 67% (–), 11% (+/–), $n = 17$) to exclusively transforming the (+) enantiomer. Soil warming in Norway, on the other hand, caused soils to shift from all samples preferentially removing the ruelene (+) enantiomer to some (22%) preferentially removing the less toxic enantiomer ($n = 13$). Technical obstacles to separating ruelene enantiomers were greater than those faced with dichlorprop; therefore, fewer soil samples were successfully analysed (17 from Brazil and 13 from Norway). Nevertheless, results were consistent with the behaviour of dichlorprop, where environmental changes affected enantioselectivity when long lag periods were required for adaptation.

Delayed microbial transformation of chemical residues is commonly observed and is often assumed to represent the amount of time required for low concentrations of microorganisms responsible for the transformation process to attain significant numbers after using the chemicals as substrates or energy sources. Hollibaugh[14], however, investigated enantioselectivity for D- and L-amino acids metabolized by marine bacteria and found that lag periods could not be explained by growth of sub-populations. Instead, they were consistent with times required for activating metabolically quiescent microbial populations or inducing enzymes for enantiomer-specific uptake or transformation processes.

As environmental changes in this study affected only the two chemicals with long lag periods, sorption, a more rapid process, probably did not play a role. Enantioselective sorption has been ruled out elsewhere as well[7]. Nevertheless, many soils have their genesis with living matter and their organic components may retain chiral centres capable of selectively binding specific enantiomers. Enantioselective sorption to soil organics, if it occurs, could affect the availability of various enantiomers for microbial transformation.

To investigate the diversity and biogeography of bacteria capable of demethylating methyl dichlorprop, small-subunit (16S) ribosomal

RNA (rRNA) genes of 50 soil bacterial isolates were compared using amplified ribosomal DNA restriction analysis (ARDRA)[15] (Fig. 2). ARDRA has proved useful in taxonomic assessments of bacterial culture collections[16] and 16S rDNA clone libraries[17] at genus and species levels.

In general, isolates from Norway and North America formed three large clusters and appeared to be more closely related to one another than to isolates from Brazil. Conversely, Brazilian isolates formed smaller clusters that were more distantly related to one another and to Norwegian and North American clusters. It is plausible that these relationships among bacterial isolates reflect ecological differences between these geographical regions. ARDRA patterns that indicated taxonomic identity between two or more isolates were, in every comparison examined except one (Norway isolates 409-1 versus 416-4), supported by 16S rDNA sequence data demonstrating gene-sequence identity. Furthermore, isolates with identical ARDRA patterns exhibited the same enantioselectivity phenotype regardless of their geographical origins, except in the case of the two isolates noted above.

Although the ARDRA banding pattern was virtually the same for Norway isolates 409-1, which preferentially transformed the (–) enantiomer, and 416-4, which was non-enantioselective, 16S rDNA sequence data varied by several base pairs. Isolate 409-1 did metabolize the (+) enantiomer, but at a much slower rate than the (–) enantiomer. In fact, with all soil samples and bacterial isolates, both enantiomers for each test chemical were transformed simultaneously, but at different rates. This behaviour is consistent with what has been observed by others where enantioselectivity has been detected in environmental samples.

Our data suggest that microbial enantioselectivity with environmental pollutants is controlled by the activation of metabolically quiescent microbial populations or the induction of enantiomer-specific enzymes, as was the case with amino acids, and that this selectivity follows lines of genetic similarity where different groups of related microbes are activated by different kinds of environmental changes.

With one exception (Norway 409-1), only isolates from Brazil exhibited enantioselectivity with methyl dichlorprop. Among those isolates, 58% were enantioselective and their preferences roughly reflected those observed with soil incubations (Table 2). However, Norwegian and North American isolates did not reflect the (–) enantiomer preference observed in soil incubations from these locations. Most of these isolates were non-enantioselective, but the relatively few genotypes that were enantioselective tended to

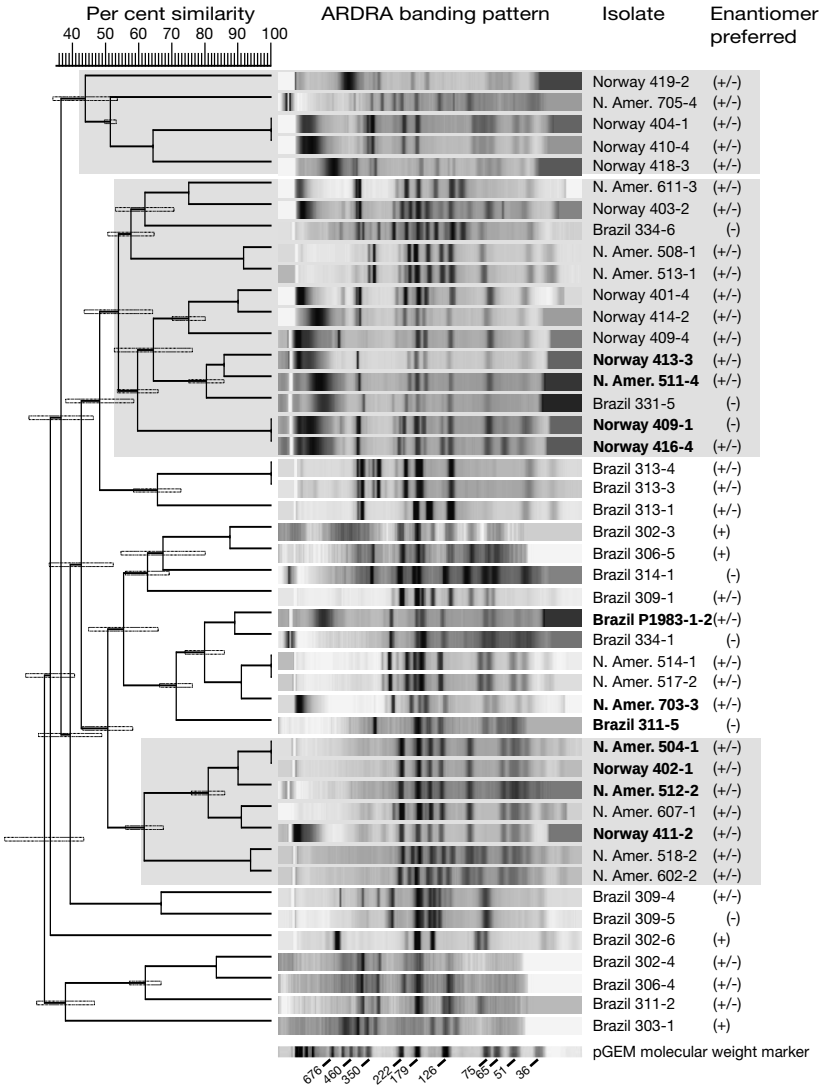

Figure 2 Amplified ribosomal DNA restriction analysis of bacterial isolates. Confidence limits for the placement of nodes are shown with error bars. Molecular weight marker (pGEM) to which band positions were normalized is shown at the bottom. Numbers indicate size in base pairs of DNA. In general, isolates that enantioselectively transformed methyl dichlorprop from North American and Norwegian soils formed three large clusters (grey boxes) whereas Brazilian isolates formed smaller, more distantly related, clusters.

dominate transformation processes such that soil samples from North America preferred either (+) or (−) enantiomers while almost all of the soil samples from Norway preferred the (−) enantiomer.

These results have important implications for public health and environmental protection. The differing effects of inorganic and organic nutrients on enantioselectivity (Tables 1, 2), for example, raise new questions with respect to land application of processed sewage sludge (biosolids), which transforms pollutants enantioselectively[18]. Under a regulation first promulgated by the US Environmental Protection Agency in 1993 (ref. 19), biosolids are used as a commercial fertilizer for agricultural lands heavily treated with pesticides. In the USA, Europe, and elsewhere, governments are promoting the agricultural use of biosolids to replace ocean dumping of municipal wastes. Substituting organic-rich biosolids for inorganic fertilizers may increase microbial transformation rates of some of the active enantiomers of pesticides and thus diminish their effectiveness in the field. On the other hand, persistence may be enhanced for some enantiomers posing a risk to public health and the environment.

This study demonstrates that significant environmental changes, such as tropical deforestation and global warming, may substantially alter the relative persistence of enantiomers of chiral pollutants, exacerbating the adverse effects of some while ameliorating the effects of others. It calls into question the accuracy and future relevance of current risk assessments for numerous pollutants, and underscores the need to better incorporate science in efforts aimed at protecting public health and the environment[20]. □

Methods

Dichlorprop and ruelene were microbially transformed rapidly enough to process numerous samples, and could also be adequately separated from extracts of soils and microbiological media. Dichlorprop, one of the most widely used herbicides, is applied as the acid and various esters that are readily attacked by common bacterial esterases. Ruelene has been banned in the USA but is still used elsewhere. Chiral Technologies (Exton, Pennsylvania, USA) separated the enantiomers from racemates and methylated the dichlorprop enantiomers.

Brazilian soil samples were collected in March and August 1998 from a forest and an adjacent 11-year-old pasture. Plots in both areas were treated with inorganic fertilizers (~33 kg N per hectare and 13 kg P per hectare). Additional samples were collected from 9-, 15-, 19-, 26- and 47-year-old pastures. Soil-warming samples with corresponding control (unheated) samples were collected in October 1998 from field plots in Harvard Forest, Massachusetts where buried, computer-controlled electrical cables heated soil 5 °C above ambient temperatures, beginning in July 1991. Samples were also collected in September 1998 from field plots in Risdalsheia, Norway that had been similarly heated since May 1994.

Soil samples of 1-gram each were treated with 10-ml aqueous racemic herbicide or insecticide solutions containing ~25 mg l^{-1} of each enantiomer and incubated at 25 °C overnight for methyl dichlorprop and ~6 months for dichlorprop and ruelene. Half-lives were estimated from changes in pollutant concentrations after transformation began, assuming first-order kinetics. Enantiomer concentrations usually decreased >80%.

Methyl dichlorprop enantiomers were detected by flame ionization (FID) after separation with a 25 m × 0.25 mm wall coated, open tubular fused silica column coated with a 0.25-μm layer of CP ChirasilDex CB (Chrompack, Ravitan, New Jersey, USA). Dichlorprop enantiomers were separated by capillary electrophoresis (CE) (15 kV with 25 mM tetraborate buffer and 25 mM trimethyl-β-cyclodextrin) and detected by ultraviolet absorption at 230 nm. Ruelene enantiomers were also separated by CE in the micellar electrokinetic chromatography mode (20 kV with 20 mM tetraborate buffer at pH 8.5 containing 100 mM sodium dodecyl sulphate, 20% acetonitrile, and 40 mM 2-hydroxypropyl-β-cyclodextrin) and detected at 200 nm.

Only samples demonstrating a decrease of ≥15% in racemates (two standard deviations of the experimental error for determining concentrations) were analysed for enantioselectivity. Samples exhibiting enantioselectivity generally had a ≥50% difference between concentrations of the two enantiomers at the final sampling time; non-enantioselective samples (+/−) generally showed no significant differences.

For soils enriched with organic nutrients, the 10-ml volume of added liquid contained various concentrations (undiluted, and diluted by factors of 100 and 10,000 of beef extract plus peptone (Difco Laboratories, Detroit, Michigan, USA), a standard source of complex organic nutrients for culturing a wide variety of microorganisms. Net preferences, which indicated the extent to which soil (or soil + organic nutrients) preferentially degraded an enantiomer, were absolute values of differences in percentage of samples preferentially degrading (+) or (−) enantiomers. All dilutions of the organic nutrients affected enantioselectivity similarly; therefore, these results were pooled in Table 2. Because Brazilian forest and pasture samples exhibited almost identical degrees of preference for methyl dichlorprop enantiomers, results from these samples were also combined in Table 1.

To operationally determine the taxonomic relationships between bacterial isolates, restriction banding patterns of the 16S rDNA of each isolate were compared. Phenol/chloroform-extracted DNA from each bacterial isolate was used as the template in a polymerase chain reaction (PCR) to amplify 16S rDNA. The primers used in the PCR (27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACG ACTT-3′)) yielded an amplicon corresponding to bases 8 through 1492 (*Escherichia coli* numbering) of the bacterial 16s rDNA. After the PCR, the 16S rDNA amplicon was purified by phenol/chloroform extraction and digested with two restriction enzymes, *Rsa*I and *Alu*I. Restriction products were separated by electrophoresis on a highly resolving 3% agarose gel (Metaphor, FMC, Rockland, Maine, USA) and visualized using a laser gel scanner and the double-stranded DNA fluorescent stain SYBR Gold (Molecular Probes, Portland, Oregon, USA). Gel lanes were normalized to a single molecular weight standard and analysed using the computer software program Molecular Analyst. Banding patterns were compared based on constituent band positions. The cluster diagram, based on differences in ARDRA banding patterns, was produced by UPGMA (unweighted pair group method using arithmetic averages) of a Jaccard similarity matrix. The cophenetic correlation was 77%. To investigate the reliability of ARDRA relationships a portion (~720 base pairs) of the 16S rDNA was sequenced from the isolates shown in bold-faced type in Fig. 2. In general, isolates with similar or identical banding patterns also had similar or identical 16S rDNA sequences.

Mortality rates with ruelene were determined by exposing North American forest tent caterpillars (*Malacosoma disstria*). Forty larvae from a single nest were divided into two equal groups and fed equal amounts of native black cherry leaves coated with either the (+) or (−) enantiomer. The insecticide was dissolved in acetone (200 mg ml^{-1}), thinly spread over the surface of the leaves and allowed to air dry. Similar tests, in which plantains (*Plantago* sp.) were treated with 700 mg l^{-1} of separate enantiomers of methyl dichlorprop, showed that, as with dichlorprop, only the (+) enantiomer is herbicidal.

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