Degradation of SDM Effluent Wastewater

![Graph showing degradation over time with two lines: 1100 and 500.]

(54) Title: MICROBIAL CONSORTIUM FOR THE BIODEGRADATION OF DITHIOCARBAMATES

(57) Abstract: The invention relates to a method for biodegrading dithiocarbamates or related compounds which are present in a contaminated environment. The method involves contacting the contaminated environment with a microbial consortium comprised of methylotrophic bacteria such as the genera of bacteria: Alcaligenes, Pseudomonas, and Hypomicrobium, and maintaining the microbial consortium in contact with the contaminated environment for a time that is sufficient for the microbial consortium to degrade the dithiocarbamates or related compounds. Other bacterium, such as Thiobacillus may optionally be present as part of the consortium.
MICROBIAL CONSORTIUM FOR THE BIODEGRADATION OF DITHIOCARBAMATES
MICROBIAL CONSORTIUM FOR THE BIODEGRADATION OF DITHIOCARBAMATES

FIELD OF THE INVENTION

This invention relates to a microbial consortium useful for biodegrading dithiocarbamates.

BACKGROUND OF THE INVENTION

Dithiocarbamates are used in a variety of water treatment applications as metal precipitating agents. Dithiocarbamates provide a cost effective means of removing heavy metals from metal processing wastewater. Dithiocarbamates, however, pose a toxicity problem. In particular, dithiocarbamates have been shown to be inherently toxic to fish and other wildlife. In addition, dithiocarbamates may with time and under certain conditions autocatalytically hydrolyse to carbon disulfide which also poses a toxicity problem.

Mounting public concern and increasing environmental legislation have provided the impetus for a safe, effective means to remediate dithiocarbamates contaminated environments. Past methods of disposing of wastewater or soil containing dithiocarbamates have included dumping at specified land-fill areas, isolation in suitable, reinforced containers, land based deep-welling, dumping in deep water at sea and incineration. All of these methods carry some potential for harm to the environment. For example, incineration creates a problem of air pollution and disposal on land risks the possibility that toxic substances will leach into locations where they may threaten aquatic life forms, animals or humans. A more desirable disposal method might incorporate a chemical, enzymatic, or biological degradative process.
The metabolic reduction of dithiocarbamates is reported. J.


These authors, however, did not demonstrate the bacterial degradation of SDM as breakdown product of tetramethyl thiuram disulfide above the levels expected in non-biological controls. One additional paper also describes the microbial degradation of TMTD (K. Maeda and T. Tomomura “Microbial degradation of tetramethyl thiuram disulfide”, (1968), Kenkyuu ha kokoku (Kaogyo Gijutsuin Biseibutsu Kaogyo Gijutsu Kenkyujo (Japan) 33(1): 1-8) and identifies the degradation products as dithiocarbamate, dimethylamine, formaldehyde, elementary sulfur, and methionine.

There remains a need for an effective degradation process for dithiocarbamates and related compounds that will degrade those compounds completely and is effective in both the in vitro and in situ remediation of contaminated environments including both soil and water systems.
SUMMARY OF THE INVENTION

Accordingly, it is an object of the invention to provide a microbial consortium capable of biodegrading dithiocarbamates.

It is another object of the invention to provide a method for biodegrading dithiocarbamates in which a microbial consortium is added to contaminated soil or water for the purpose of biodegrading dithiocarbamates present as contaminants in the soil or water.

It is also an object of the invention to provide a method for biodegrading dithiocarbamates to substances which are environmentally safe.

With regard to the foregoing and other objects, the present invention provides a method for biodegrading dithiocarbamates or related compounds which are present in a contaminated environment, said method comprising contacting the contaminated environment with a microbial consortium comprising methylotrophic bacteria including a number of bacteria such as Alcaligenes, Pseudomonas, Hypomicrobium, and other methylotrophs, and optionally other bacterium such as Thiobacillus, maintaining the microbial consortium in contact with the contaminated environment for a time that is sufficient for the microbial consortium to degrade the dithiocarbamates or related compounds.

For its use in the course of decontamination of contaminated soils and waters, the consortium is applied to environments having a pH-value of between 5.0 and 8.5 and in a temperature range of from 5°C to 42°C. The consortium is applied in a quantity that results in a final soil concentration of greater than $10^6$ cells per gram of contaminated soil. In water, the consortium is applied at a level resulting in a concentration of greater than $10^6$ cells/ml.. The amount of consortium containing material applied would depend on the density of cell mass in the consortium preparation, and this can be adjusted by dilution or concentration techniques. Likewise, the consortium may be applied in a dried mass absorbed to carrier particles such as shredded waste agricultural stock.
The process for its use includes basically the one-time or several-time spraying (dissolved in water) or spreading of the mixture (dried) on to the contaminated mass and afterwards the optimization of the milieu by aeration or nutrient addition. For waste stream purification, the process includes developing the consortium on a solid matrix such as gravel or other appropriate fill material and optimizing the flow of the waste stream through the bed to achieve degradation.

According to another aspect the invention provides a microbial consortium comprised of multiple genera of bacteria consisting of *Pseudomonas* and *Hyphomicrobium* species with secondary amounts of, *Alcaligenes* and other species of facultative methylotrophs. The consortium could optionally contain other bacteria, such as *Thiobacillus*. In one preferred embodiment, the consortium contains *Thiobacillus* bacteria. The microbial consortium has been isolated from a contaminated environment and is capable of biodegrading dithiocarbamates or related compounds.

The microbial consortium when applied to a contaminated environment biodegrades dithiocarbamates and related compounds to intermediates such as sulfide and dimethylamine which are then oxidized to carbon dioxide, water, ammonia, and sulfate. The microbial consortium is effectively applied to soil, water, treatment ponds, treatment ditches, waste disposal sites, and waste streams which are contaminated with dithiocarbamates or related compounds.

**BRIEF DESCRIPTION OF THE DRAWING**

Figure 1 shows the percent degradation of dithiocarbamate by the consortium over time.

**DETAILED DESCRIPTION OF THE INVENTION**

The invention relates to a novel microbial consortium capable of biodegrading dithiocarbamates and related compounds. The
dithiocarbamates or related compounds are present as contaminants in the environment. As used herein, "contaminated environment" or "contaminated environments" means any environment contaminated with dithiocarbamates or related compounds. Typical contaminated environments may include, but are not limited to, soil, water, treatment ponds, treatment ditches, manufacturing facilities, waste disposal sites, and waste streams.

The dithiocarbamates may be in the form of a liquid, solid or combination thereof. The dithiocarbamates include, but are not limited to sodium diethyl dithiocarbamate, sodium dimethyl dithiocarbamates, sodium dipropyl dithiocarbamates, and sodium dibutyl dithiocarbamates.

ISOLATION OF AND COMPOSITION OF THE CONSORTIUM

As used herein, "microbial consortium" refers to any collection of microorganisms which are capable of biodegrading dithiocarbamates.

The microbial consortium of the present invention was isolated from a waste treatment facility of an industrial site and selected by the following method. A sample of soil was inoculated into minimal medium (buffered mineral salts) supplemented with 100 ppm of sodium dimethyl dithiocarbamate, 500 ppm of dimethyl amine, vitamins and yeast extract, and incubated for 7 days at 25°C. The culture was further subcultured into minimal medium supplemented with 250 ppm of sodium dimethyl dithiocarbamate, 250 ppm of dimethyl amine, vitamins and yeast extract, and incubated and additional 7 days at 25°C. The culture was further subcultured into minimal medium supplemented with 500 ppm of sodium dimethyl dithiocarbamate, 100 ppm of dimethyl amine, vitamins and yeast, and incubated until visible cellular turbidity as observed at 25°C. The above process was repeated an additional two times until the final enrichment contained sodium dimethyl dithiocarbamate at 1000 ppm and no dimethylamine.
The concentration of sodium dimethyl dithiocarbamate was monitored during the isolation procedure by a colorimetric assay which involved forming a copper-sodium dimethyl dithiocarbamate complex and measuring color formation. The assay involved combining 1 ml of water, 1 ml of copperacetate (0.103 g/100 ml) and 2 ml of sample. Color development was determined by measuring absorbance at 430 nm. A reduction in absorbance indicated removal of dithiocarbamate. One isolated group of bacteria, herein called the microbial consortium, proved to rapidly reduce the concentration of dithiocarbamate and has maintained this ability through a number of successive transfers indicating the consortium has the ability to degrade dithiocarbamate and that this is a stable trait.

Analysis of the members of the consortium has been completed by a number of approaches. Several methods indicated that there were at least four and as many as 7 different bacterial types present in the consortium including the species Alcaligenes, Pseudomonas, and Hypomicrobium. Only the Alcaligenes spp. and the Pseudomonas spp. are culturable using conventional culturing techniques. The presence of the putative Hypomicrobium spp. was determined by visual microscopic (Hypomicrobium morphology) and molecular biology techniques.

Since it is difficult, at best, to accurately characterize a mixed consortium of microorganisms, several different approaches had been utilized. The difficulty in characterizing environmental microorganisms is well know in the field and results from two general observations, 1) many bacteria cannot be cultured by themselves in the laboratory although they may be repeatedly grown in a complex mixture (consortium), and 2) describing a particular bacterium as a precise “species” is not always possible and there may not be a absolute designation. In bacteria, the concept of species is much debated (see J.T. Staley (1999), ASM News vol. 65(10) 681-687).

Therefore the following descriptions of the members of the consortium must consider this.
Standard bacteriological plating techniques on different growth media have revealed that the majority of culturable bacteria in the consortium could be assigned to the genus *Pseudomonas/Hypomicrobium*, and *Alcaligenes*. Since plating on bacteriological medium generally only reflects a fraction of an environmental population, the consortium has also been analyzed using molecular techniques. Purified DNA from the consortium was subjected to the polymerase chain reaction using primers specific for the 16S rRNA genes. The amplified genes were then separated and analyzed using density gradient gel electrophoresis (DGGE) as described by Muyzer et al., 1993 (Appl. Environ. Microbiol. 59, 695-700). Each band on the resulting gel indicates a unique microorganism. DGGE results suggest that there are a minimum of 4 and a maximum of 7 unique bacteria in the consortium. Most of these appear to be *Pseudomonas*, *Hyphomicrobium*, and *Alcaligenes*. Phase contrast microscopy has also confirmed that some members have a characteristic *Hyphomicrobium* morphology. Finally, many types of bacteria have unique signature fatty acids. The total lipid from the consortium were extracted, separated by polarity using column chromatography and the fatty acid fraction purified, derivatized and characterized by gas-liquid chromatography. This analysis confirmed the presence of methanol-utilizing *Pseudomonas* and *Hyphomicrobium* spp. The results are consistent of the Type B methylotrophs of Urakami and Komagata (J. Gen Appl. Microbiol., 25: 343-360 (1979) which include *Pseudomonas*, *Hyphomicrobium*, *Methylobacillus*, *Acetobacter*, and *Xanthomonas* and others.

In summary, the consortium is a mixture of mostly methylotrophic bacteria dominated by *Pseudomonas*, *Alcaligenes*, and *Hyphomicrobium*. Other methylotrophs and *Thiobacillus* species may be present in minor amounts. No single culturable member of the microbial consortium has demonstrated the ability to biodegrade dithiocarbamates when inoculated into medium containing sodium dimethyl dithiocarbamate as sole carbon source. It appears that it is necessary for a microbial consortium of at least several type
B methylo trophic microorganisms together for complete dithiocarbamate
degradation to occur.

This consortium has been deposited with the American type Culture
collection under the terms of the Budapest Treaty and has been assigned the
assession number as ATCC XXXXX.

For its use in the course of decontamination of soils and waters, the
consortium is best prepared in a synthetic medium (comprised potassium
phosphate buffer 0.02M (pH 6-8), ammonium nitrate (0.5g/l), potassium
chloride (0.25g/l), magnesium sulfate heptahydrate (0.25g/L) and between
500 and 2000 ppm SDM and between 100 and 1000 ppm dimethylamine. It
is best grown between 20-35°C, the cultivation time between 4 to 7 days
depending on the requirements of the density of the bacterial suspension. It
may then be lyophilized (freeze dried) or prepared as a concentrated slurry by
removal of water.

The preparation may be used by spraying or spreading on to
contaminated soil or by inoculation into contaminated water. The method also
allows containment of the contaminated soil or water in a bioreactor (tank)
followed by inoculation with the consortium. The method further allows
establishment of an attached consortium on a solid substrate such as gravel
and thereby establishing a flow through reactor where the flow rate is
regulated to maximize degradation of the dithiocarbamate. In all cases the
microbial mixture is added in a quantity in which the final cell density is
greater than about 1.0 x 10^6 consortium members per gram of soil or per ml
of water. Lesser amounts may be initially applied, and the number of cells
allowed to increase though cell growth, however the rate of degradation will
be significantly slower. A preferred level of consortium members is about 10^7
per gram of soil or per ml of water. The pH value of the treated medium
preferably is kept between 5.0 and 8.5 and the degradation process is
continued under aerobic conditions.

It has been found that preparations of the consortium generally
contain about 10^8 cells/ml. Thus to treat 100 liters of water, with a desired
treatment level of $10^7$ cells/ml, one would add 10 liters of the consortium having $10^9$ cells/ml to 90 liters of water. If the concentration of the consortium is higher than $10^8$/ml, then a lesser treatment could be used. For a faster rate of degradation, a greater volume of consortium could be used. It has been found that generally about 10 to 20 percent (volume/volume) of the consortium to aqueous system is an appropriate treatment level.

An example of a treatment of a soil system would be the treatment of a contaminated soil of one square meter contaminated to a depth of 6 inches. This would represent approximately 152,400 cm$^3$. To obtain a treatment level of $10^7$ cells per gram of soil, and assuming a dried consortium cell concentration of about $10^{11}$ cell/g, about 22 grams of dried consortium would be required for this square meter.

The result of the application of the inventive mixture of natural microorganisms is a degradation of the dithiocarbamate structure in the environment to mostly carbon dioxide and cell mass.

The advantages of the consortium and its uses are to be seen in the fact that the created metabolites are not toxic. The only potential toxic byproduct of metabolism is carbon disulfide and/or hydrogen sulfide, however, degradation of carbon disulfide to carbon dioxide is the putative role of the Thiobacillus spp. in the consortium as is known (S. L Jordan, A. J. Kraczkiewicz-Dowlat, D.P. Kelly, and A.P. Wood, "Novel eubacterium able to grow on carbon disulfide (1995), Arch. Microbiol. 163: 131-137). Also a number of microorganisms (sulfur oxidizing) can oxidize hydrogen sulfide. While the exact mechanism of degradation is not known, it is very likely that the first step in the degradation of the dithiocarbamates is due to the oxidation of one of the sulfur groups by a consortium containing monoxygenase. When this occurs, at neutral pH, one of the sulfurs is released as a sulfide and the remaining part of the dithiocarbamate structure becomes cell associated and is eventually assimilated into cell mass. The ability to do this is specific to this consortium in that a number of other bacteria and
enrichments have been evaluated and no enzyme mediated oxidations are observed.

A further advantage is that the number of beneficial soil microorganisms in the soil is increased improving the structure of the soil and converting the carbon, nitrogen, and sulfur entrained as dithiocarbamate back to inorganic elements for use by plants and soil microorganisms. In view of the fact that the organisms used are isolated from the natural environment and that they are not genetically engineered, there is no danger for a negative influence on the biosphere whatsoever.

The following nonlimiting examples illustrate further aspects of the invention.

EXAMPLES

The following materials and methods were used in the Examples:

1. Sodium dimethyl dithiocarbamate (SDM) or dimethyl dithiocarbamate acid, sodium salt hydrate was obtained from Fluka Chemical as a 40% solution in water.

2. Sodium Dipropyl dithiocarbamates and Sodium Dibutyl dithiocarbamates were synthesized at Alco Chemical Corporation.

Spectrophotometric determinations were performed using a Beckman DU-6 spectrophotometer.

EXAMPLE 1

Biodegradation of SDM in aqueous systems.

A 1000 ml bioreactor containing the synthetic medium (described above) has been inoculated with the consortium density of approximately 1.1 x 10^7 ml. The initial pH value was 7.2 and the temperature varied between 20 – 27°C. The consortium density was measured by direct epifluorescent microscopic counts using the DNA specific dye 4,6-diamidino-2-phenylindole.
SDM concentration was measured spectrophotometrically by complexing with cupric acetate and measuring the absorbance at 430 nm.

**TABLE I**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cell Count x10⁶ Average</th>
<th>STD</th>
<th>Consortium added SDM (mg/L)</th>
<th>Control (w/o consortium SDM (mg/ml))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>111</td>
<td>6.4</td>
<td>988</td>
<td>959</td>
</tr>
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<tr>
<td>168</td>
<td>1071</td>
<td>103</td>
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<td>812</td>
</tr>
<tr>
<td>312</td>
<td>933</td>
<td>85</td>
<td>&lt;20</td>
<td>794</td>
</tr>
</tbody>
</table>

**EXAMPLE 2:** Degradation of an industrial waste effluent-

To further demonstrate the degradation of dithiocarbamate by the consortium, an actual waste sample, waste wash-water, from an industrial production plant was evaluated. The sample contained about 1100 ppm of SDM. The sample, and a dilution of it at 500 ppm and pH 7.8, was inoculated with a 20% (V/V) amount of a 72 h consortium. A control without inoculation was included. The results shown in Figure 1 show that the consortium degraded almost 100% of the SDM within a 72 hour period.

**EXAMPLE 3**

Biodegradation of Sodium Dimethylthiocarbamate by Microbial Consortium in Soils

Two one-kilogram soil samples were contaminated with from 100 to 5000 mg of SDM. One soil environment was inoculated with approximately 1.2 X 10⁷ consortium members/g soil while the other remained uninoculated. Nitrogen and phosphorus was added to both systems. The initial soil pH was 6.8 and the temperature varied from 18°C to 27°C. Water was added to maintain the dry weight at about 30%. Each soil system was mixed weekly to provide aeration. At the indicated periods, soil aliquots were extracted with a methanol:chloroform:water (1:1:0.9) and the SDM concentration determined as indicated above.
TABLE II
PERCENT degradation of SDM

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>100</th>
<th>500</th>
<th>1000</th>
<th>2000</th>
<th>5000</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.6</td>
<td>6.2</td>
<td>4.8</td>
<td>3.9</td>
<td>5.2</td>
</tr>
<tr>
<td>3</td>
<td>18.3</td>
<td>22.6</td>
<td>16.3</td>
<td>12.4</td>
<td>3.1</td>
</tr>
<tr>
<td>7</td>
<td>50.2</td>
<td>63</td>
<td>39.7</td>
<td>26.7</td>
<td>2.3</td>
</tr>
<tr>
<td>10</td>
<td>81</td>
<td>88</td>
<td>48</td>
<td>32.1</td>
<td>1.8</td>
</tr>
<tr>
<td>14</td>
<td>87.3</td>
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<td>1.8</td>
</tr>
<tr>
<td>28</td>
<td>92.4</td>
<td>89</td>
<td>81</td>
<td>58</td>
<td>3.2</td>
</tr>
</tbody>
</table>

% degradation was determined by comparison with uninoculated controls

5 EXAMPLE 4

The following example demonstrates other N-alkyl dithiocarbamates that can also be degraded by the consortium.

A 1000 ml bioreactor containing the synthetic medium (described above) has been inoculated with the consortium density of approximately 1.1 x 10^7 ml. The initial pH value was 7.2 and the temperature varied between 20–27°C. Dithiocarbamate concentration was measured spectrophotometrically by complexing with cupric acetate and measuring the absorbance at 430 nm.

The results show that the consortium is capable of degrading a variety of N-alkyl dithiocarbamates to varying degrees.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% Diethyl Dithiocarbamate</th>
<th>% DiPropyl Dithiocarbamate</th>
<th>%Dibutyl Dithiocarbamate</th>
<th>% Dimethyl Dithiocarbamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.28</td>
<td>3.99</td>
<td>4.44</td>
<td>3.02</td>
</tr>
<tr>
<td>0.5</td>
<td>2.80</td>
<td>3.87</td>
<td>4.70</td>
<td>8.1</td>
</tr>
<tr>
<td>1.5</td>
<td>8.78</td>
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<td>9.5</td>
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<tr>
<td>4</td>
<td>8.18</td>
<td>3.56</td>
<td>3.81</td>
<td>12.3</td>
</tr>
<tr>
<td>5</td>
<td>13.29</td>
<td>3.49</td>
<td>3.98</td>
<td>12.1</td>
</tr>
<tr>
<td>7</td>
<td>15.01</td>
<td>3.37</td>
<td>4.12</td>
<td>27.1</td>
</tr>
<tr>
<td>27</td>
<td>18.00</td>
<td>7.82</td>
<td>6.11</td>
<td>61.4</td>
</tr>
<tr>
<td>51</td>
<td>21.00</td>
<td>9.43</td>
<td>6.66</td>
<td>77.8</td>
</tr>
<tr>
<td>73</td>
<td>23.00</td>
<td>11.24</td>
<td>7.93</td>
<td>88.7</td>
</tr>
<tr>
<td>96</td>
<td>25.07</td>
<td>14.56</td>
<td>9.46</td>
<td>97.5</td>
</tr>
</tbody>
</table>

*% degradation is calculated as percent loss over the abiotic Control

-13-
WHAT IS CLAIMED IS:

1. A microbial consortium comprised of methylotrophs comprising the bacterial genera *Alcaligenes, Pseudomonas, and Hypomicrobium*, said microbial consortium being capable of biodegrading dithiocarbamates or related compounds.

2. The microbial consortium of claim one wherein said consortium comprises 40 to 70 percent *Pseudomonas*, and *Hypomicrobium* bacterium and from 20 to 40 percent *Alcaligenes* bacterium.

3. The microbial consortium of claim 1 further comprising *Thiobacillus* bacteria.

4. A method for biodegrading dithiocarbamates or related compounds which are present in a contaminated environment, said method comprising contacting the contaminated environment with a microbial consortium comprised of methylotrophic bacteria comprising *Alcaligenes, Pseudomonas,* and *Hypomicrobium* bacterium and maintaining the microbial consortium in contact with the contaminated environment for a time that is effective for the microbial consortium to degrade the dithiocarbamates or related compounds.

5. The method of claim 4 wherein the concentration of the microbial consortium in contact with said contaminated environment is greater than $10^6$ cells per gram of contaminated soil, or $10^6$ cells per milliliter of contaminated water.

6. The method of claim 5 wherein the concentration of the microbial consortium in contact with said contaminated environment is greater than $10^7$ cells per gram of contaminated soil, or $10^7$ cells per milliliter of contaminated water.
7. The method according to Claim 4 wherein the microbial consortium is produced by culturing a naturally-occurring population of microorganisms in a medium comprising dithiocarbamates.

8. The method according to Claim 4 further comprising the step of adding to the contaminated environment a member selected from the group consisting of a nutritional source of nitrogen and a nutritional source of phosphorous for the microbial consortium.

9. The method according to Claim 4 further comprising the step of adding water to the contaminated environment.

10. The method according to Claim 4 wherein the contaminated environment is a member of the group consisting of a water and an aqueous slurry of soil or other particulate matter.

11. The method according to Claim 4 wherein the contaminated environment is maintained at a pH within a range of 5.0 to 8.5.

12. The method according to Claim 4 wherein the contaminated environment is maintained at a temperature within a range of 5°C to about 42°C.

13. The method according to Claim 4 further comprising the step of sequestering the contaminated environment in a vessel.
Degradation of SDM Effluent Wastewater

% Degradation (over control)

Time (h)

- 1100
- 500
### A. CLASSIFICATION OF SUBJECT MATTER

IPC 7  COF3/34  C1P39/00  B09C1/10  //COF101:38

According to International Patent Classification (IPC) or to both national classification and IPC.

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7  C12P  B09C  CO2F  C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>WARTON BEN ET AL.: &quot;The soil organisms responsible for the enhanced biodegradation of metham sodium&quot; BIOLOGY AND FERTILITY OF SOILS, vol. 34, no. 4, September 2001 (2001-09), pages 264-269, XP009011728 the whole document ---</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

### Date of the actual completion of the international search

3 June 2003

### Date of mailing of the international search report

17/06/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentilaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epc nl Fax: (+31-70) 340-3016

Authorized officer

Borello, E
INTERNATIONAL SEARCH REPORT

Box I  Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. X Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
   see FURTHER INFORMATION sheet PCT/ISA/210

3. □ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II  Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. □ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. □ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

□ The additional search fees were accompanied by the applicant’s protest.

□ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)
Continuation of Box I.2

Present claims 1 and 4 relate to an extremely large number of possible compounds. In fact, the subject-matter of claims 1 and 4 referring to "... or related compounds..." contains so many options that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible.

Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), namely the biodegradation of dithiocarbamates, as disclosed and supported in the examples 1-4.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.
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