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Corso di Laurea Specialistica in Ingegneria per l'Ambiente e il Terrirorio

Curriculum Protezione e Risanamento dell'Ambiente

Qualifying and quantifying linuron degradation capacity with a colorimetric reaction? Test case on a linuron treated agricultural soil.

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-ANNO ACCADEMICO 2009-2010-

Summary

1. Introduction

Herbicides are one of the main categories of crop protection products and they kill weeds and other plants that grow where they are not wanted. Among these substances there are the phenylurea herbicides that are a group of herbicides used mainly in either pre- or post emergence treatment of cotton, fruit, cereal, or other agricultural crops. This group of herbicides was introduced shortly after Second World War and became one of the most important classes of crop protection products. In recent years researchers are paying greater attention to this family of herbicides because of their high biotoxicity and possible carcinogenic properties and they require several weeks to months for their removal from the environment. Some of these phenylurea herbicides (isoproturon and diuron) are considered as priority hazardous substances by the Water Framework Directive of the European Commission, and are included in Decision No. 2455/2001/EC of the European Parliament and of the Council of November 20th 2001, in which a list of priority substances in the field of water policy is established.

The phenylurea herbicides include widely used compounds such as diuron, isoproturon (IPU) and linuron. Phenylureas are considered to be moderately persistent in the environment. Experiments in laboratory for different agricultural soils incubated at 10–20 °C have shown values ranging from a few days up to several years. Some of this phenylureas, such as IPU, are frequently detected in ground and surface waters in concentrations exceeding the drinking water limit of 0.1 μg L^{-1} for pesticides set by the European Community (European Union, 1998). The EU has included linuron as well as diuron in a list comprising endocrine-disrupting chemicals which are suspected of interfering with hormone systems of humans and wildlife, causing birth defects, sexual abnormalities and reproductive failure in offspring (EC COM(2001)262). Environmental effects of linuron mainly concern aquatic ecosystems since linuron has been shown to be highly toxic to aquatic plants and invertebrates. Ecotoxicological data suggest that IPU and some of its metabolites are harmful to aquatic invertebrates, freshwater algae and microbial activity.

The phenylurea herbicides can be photodegraded, hydrolysed and biodegraded and persist for periods ranging from days to weeks. They are mobile in soil. The environmental fate of these herbicides in soil, is influenced by several factors such as agricultural practices, environmental characteristics, texture and hydrology condition of the soil. Degradation products of phenylureas such as 3,4-dichloroaniline (3,4-DCA) have been detected in groundwater and surface water. These metabolites may be even more persistent and bind strongly to soil constituents. The 3,4-DCA (derived from linuron or diuron) displayed a much higher toxicity than their mother compounds. Biodegradation is recognized as the primary force in transformation and mineralization of phenylurea herbicides. The rate of pesticide biodegradation does not remain constant with time, and is dependent on the physico- chemical properties of the soil and of the pesticide as well as on

the biology of the soil. Prolonged or repeated contact between soil microbes and pesticides has been shown to result in an increase in the rate and extent of biodegradation. There are many strains or consortia of strains that were isolated in recent years and which are able to degrade all or part of these herbicides. In particular, the genus Variovorax seems to play a key role in *in situ* linuron degradation. In fact several research groups have isolated linuron degrading *Variovorax* strains and previously studies showed an increase in the *Variovorax* fraction of the bacterial soil community after intensive and continuous linuron treatment during 10 weeks.

Several tests can be used to see if there is degradation capacity for herbicides present in soil. The most important techniques are mineralization and degradation experiments. Both techniques can be used to analyze the dynamics of the herbicide degradation capacity of the microbial soil community. Degradation and mineralization experiments use a minimal medium with as only carbon source the component of which we want to detect the presence or absence of degradation capacity. So the phenylurea herbicides can be added to the medium as carbon sources for indigenous soil bacteria. In the degradation experiments the soil bacterial community is added to the medium, as a small amount of soil or a salt containing soil-extract, and, periodically, quantitative analysis of the phenylurea herbicides and their metabolites is performed using a high performance liquid chromatography (HPLC). The results of degradation experiments are usually compared with the data obtained from mineralization experiments. Mineralization is the complete degradation of an organic compound to CO_2 and H_2O under aerobic conditions, and CH_4, CO_2 and H_2O under anaerobic conditions. In mineralization experiments a ^{14}C-labeled compound is used that it is added to the medium. Mineralization means extensive metabolic degradation of a compound during which the compound is used as carbon- and energy source to grow (create biomass) and some of the carbon is oxidized with release of the appropriate amount of $^{14}CO_2$. This amount of $^{14}CO_2$ is detected with appropriate instruments such as a beta-radiation counter. So through a mineralization experiment it is possible to see if the degradation which takes place in the soil is metabolically (micro-organisms actively degrade it and use it to grow on) or if they only cometabolically degrade it, in which case no ^{14}C will be found back in the respiration-CO_2. The main characteristic of mineralization is the lag time. The lag time is the time before mineralization starts, with other words, it is the time before the exponential increase in the mineralization curve begins. By comparing the two tests it is possible to see if there was complete mineralization of herbicides or if there was accumulation of some of their metabolites in the medium.

2. Research questions

The main aim of this study was to see if it is possible to replace these two techniques with a facile and sensitive colorimetric method. The color reaction introduced by Pease et al. (1962) was used. In this reaction the diazotization is followed by a coupling reaction to convert the aniline compound to an azo-dye. The degradation capacity of soil bacterial community and the herbicides degradation can be analyzed by detection and quantification of metabolites. In this study, the colorimetric test was tested to see if it is capable to detect and / or quantify the metabolites of isoproturon, linuron and diuron. This was tested on

aniline compounds by testing the diazotization-coupling reaction on this metabolite, in fact all this herbicides have an aniline compound as metabolite: 3,4-DCA for linuron and diuron, 4-isopropyl-aniline (4IA) for isoproturon. However, not for every herbicide the metabolite was available. Therefore we wanted to test the possibility to create the metabolite by chemical hydrolysis of the herbicide. In the same way we wanted to see if we could create the metabolite by means of microbial degradation.

In the second part of this study a case study on linuron treaded agricultural soil was analyzed. The soil used was taken from a potato field in Halen (Belgium) with a history of linuron treatment. Nine soil microcosms were set up in glass columns and after one week of adaptation to the incubation conditions, different irrigation treatments were started. The soil microcosms were subjected to different treatments, with three replicates for every treatment: (1) a discontinuous irrigation with sterile tap water (this treatment is designated as H_2O), (2) a discontinuous irrigation with sterile tap water containing linuron (50 mgL^{-1}) (this treatment is designated as ∞ x LIN), (3) a single addition of 1mL of 50 mgL^{-1} linuron in sterile tap water at the start of different treatments, followed by discontinuous irrigation with sterile tap water (this treatment is designated as 1x LIN). Just before and after 28 days of incubation under different linuron irrigation regimes small amounts of soil were taken from the surface of the soil column and mineralization and degradation experiment (3,4-DCA production monitored by HPLC and the colorimetric test) were started. In this way the diazotization-coupling colorimetric reaction to monitor degradation capacity by monitoring the production of 3,4-DCA was tested in a practical set-up. After four weeks of different linuron treatments soil samples were taken for DNA-extraction. Soil DNA-extracts were used to perform a molecular analysis of the *Variovorax* population (size and composition) and of the linuron hydrolysis gene pool. This because it has been hypothesised before that the genus *Variovorax* plays an important role *in situ* linuron degradation.

3. Results

The hydrolysis test was performed by using a solution of strongly alkaline medium (NaOH) and the herbicides. After hydrolysis the presence of metabolites can be observed by performing the colorimetric test. The result obtained for each herbicide was that the hydrolysis of these compounds does not work in these conditions, and no aniline-metabolite was formed. Unable to create the metabolites by hydrolysis it was tried to create it through microbial degradation. This experiment was conducted using the strain *Sphingomonas* SRS2 for IPU and strain *Variovorax* SRS16 for diuron and linuron. Only for the diuron-degradation test, the minimal medium was supplemented with 1% of yeast as suggested by Sorensen et al. (2008). The quantitative analysis of metabolites was performed using HPLC and the coupling-diazotization reaction. During this experiment it was noted that the yeast extract interferes with coupling-diazotization reaction. Since the degradation of diuron by *Variovorax* SRS16 can only be performed in the presence of yeast and there was no access to another diuron degrading strain, it was decided to not continue the microbial degradation tests on diuron. The test was not continued also for IPU. In this case, in fact, it was seen that no color was developed although HPLC-results showed degradation of IPU. By analyzing the degradation

pathway of IPU by *Sphingomonas* SRS2 proposed by Sørensen et al.(2001), which does not result into formation of an aniline compound, but results into metabolites that cannot be detected with color reaction proposed by Pease (1962). In the case of linuron, instead color was developed from color reaction. The concentration of 3,4-DCA was calculated with a standard curve for 3,4-DCA. This standard curve was created measuring A_{550} (absorbance) of solutions of the product of the diazotization-coupling reaction of known concentrations of 3,4-DCA in water. From this standard curve it is possible to obtain the unknown concentration values of 3,4-DCA in different samples by measuring A_{550}. The concentration of 3,4-DCA obtained from the color reaction was found to be higher than that obtained by HPLC with differences between 5.6% and 29.5%. Because of this high variation and deviation from the trustworthy HPLC-quantification we can conclude that this technique is not accurate enough to observe linuron degradation through the production of 3,4-DCA.

For use of the colorimetric reaction for detection of 3,4-DCA in degradation experiments with environmental samples a standard curve of 3,4-DCA in this soil extract was made to see if the organic matter that was dissolved in the soil suspension, would interfere with the colorimetric reaction. Although very little dissolved organic matter remained in the soil suspension, a standard curve for 3,4-DCA was made in the soil suspension, to be able to quantify 3,4-DCA as accurately as possible in soil suspension. Mineralization capacity was evaluated in the soil microcosms before the start of the different linuron irrigation treatments and after 4 weeks of incubation under different linuron irrigation regimes. Before the start of the treatments within 32 days an average value of 38.9 % ± 3.5% of the added ^{14}C-linuron was metabolized to $^{14}CO_2$ by the soil microbial community in all samples. The average lag time was 6.91 ± 0.28 days. By analyzing the results obtained from the mineralization experiment after the different irrigation treatments, we can see a significant difference ($P < 0.05$) in the amount of ^{14}C-linuron metabolized to $^{14}CO_2$, depending on the linuron irrigation regime. In soil samples treated with a discontinuous irrigation with linuron, within 32 days of incubation, 46.7 % ± 0.76% of the added ^{14}C-linuron was metabolized to $^{14}CO_2$. The percentage of the added ^{14}C-linuron that was metabolized to $^{14}CO_2$ in the other two treatment within 32 days is 34.4% ± 3% for soils not treated with linuron and 38.9% ± 1.75% for a single addition of linuron. By comparing the results from the mineralization experiments before and after the irrigation treatments, a difference in the mineralization capacity of the three different treatments was seen. In soil with discontinuous irrigation with linuron after 32 days of incubation the 46.7 % ± 0.76% of the added ^{14}C-linuron was metabolized to $^{14}CO_2$, instead before treatment in the same period only the 37.03 ± 3.34% was metabolized. In water treatment after 32 days of incubation it was observed a decrease in mineralization capacity in fact the value decreased from 43.03% ± 0.4% to 34.4% ± 3%. A little increase of mineralization capacity, between the two sampling times, was shown in soil with a single addition of linuron. Both mineralization curves after 32 days tend to a plateau of about 37% of ^{14}C-linuron metabolized. After four weeks of this linuron treatment a decrease of lag time from 6.91± 0.28 days to 3.07 ± 0.06 days was recorded. The results show the

increase of linuron mineralisation capacity of the microbial community in a soil previously treated with linuron, in relation to the amount of linuron applied, as observed by Bers et al. (*In Press*).

Simultaneously with the mineralization experiment the degradation of linuron and the production of its metabolite 3,4-DCA by the soil microbial community, were evaluated in the degradation experiment with HPLC. The linuron concentration (obtained by HPLC) of the degradation experiment performed with the soil microbial communities before the start of the different irrigation regimes show a more or less constant concentration of linuron in the first 9 days of observation of the degradation experiment. Between day 9 and day 28 complete degradation of linuron and its metabolites took place. However, no sampling was performed in this period. Also the presence of 3,4-DCA could be monitored by the HPLC. The colorimetric reaction however, showed the presence of considerable amounts of 3,4-DCA (0.15-3.88 mg L^{-1}), even when no 3,4-DCA was detected by HPLC (detection limit of 0.1 mg L^{-1}) or degradation of linuron was already completely performed. The linuron concentrations as determined by HPLC for the degradation experiment performed with the soil microbial communities after 28 days incubation under different irrigation treatments show again in the first 9 days of measurements a constant trend in all treatments. A little decrease in linuron concentration appears to be between day 9 and day 11 although in these two days the HPLC did not detect significant traces of 3,4-DCA. This development trend is only obtained maybe due to variability in measurement of HPLC. This trend could be explained also by the fact that the soil microbial community immediately degrades 3,4-DCA so it can not accumulate. This would also mean that even if the colorimetric reaction for detection of 3,4-DCA would work well, it would not be usefull for detection of linuron degradation. The color reaction has given also in this case the concentration values of 3,4-DCA almost always higher than those measured by HPLC. However in both cases the colorimetric-quantification is too high when compared to HPLC-quantification. Based on the results, a big difference between HPLC and color reaction was observed. Since HPLC-quantification is an established and trustworthy method we can therefore conclude that the colorimetric reaction is not trustworthy for the quantification of 3,4-DCA and thus for the detection of linuron degradation.

We can hypothesise that the increase of linuron mineralisation capacity of the soil microbial community is due to the increase of the linuron degrading microbial community. Because the genus *Variovorax* is believed to play a key role in *in situ* linuron degradation, a molecular analysis was performed to have a better view on *Variovorax* community size and composition. From molecular analysis of the *Variovorax* population a difference in community composition could be detected between the microcosms which received a discontinuous supply of linuron and the microcosms which received no or a single linuron treatment. DGGE results showed 6 dominant *Variovorax* populations for the soil microcosms which were not or only once treated with linuron. Two of these populations corresponded to known populations previously associated with linuron degradation. Representatives of the most dominant population of these two, has previously been isolated out of this soil. One population corresponds to a known *Variovorax* population previously associated with not linuron degrading strains. A discontinuous treatment with linuron made the *Variovorax* community

expand to 7 dominant populations. We can hypothesise that this strain is involved in *in situ* linuron degradation.

The number of *Variovorax* 16S rRNA gene copies, measured after 4 weeks of incubation by q-PCR, is slightly different in the three irrigation treatments. After this incubation period no significant difference in the number of *Variovorax* was observed, as it was obtained by Bers et al. (*In Press*). Bers et al. only observed a significant increase (increased by about 152 times during the incubation period) in the *Variovorax* community size after 10 weeks of discontinuous linuron tretament. Also the size of bacterial community in each soil microcosms was measured to see if the results obtained for the number of *Variovorax* are influenced by variation in total bacterial population. However the size of bacterial community in the different treated soils is approximately the same. So we can conclude that the lack of a significant trend of the amount of *Variovorax* 16S rRNA gene copies in different treated soils is not due to difference in total bacterial community.

The linuron hydrolase gene could be detected in very low amounts, below the quantification limit of the linuron hydrolase specific qPCR. Therefor no accurate quantification of the amount of linuron hydrolase in the soil microcosms under different irrigation treatments could be performed. Based on these data no difference in the amount of linuron hydrolase genes could be detected, but it can not be excluded that there is a difference in the amount of linuron hydrolase genes between the different treatments.

4. Conclusions

This study showed the impossibility to replace the HPLC-measurement of linuron degradation by the diazotization-coupling colorimetric reaction. In fact, always the colorimetric-quantification was too high when compared to HPLC-quantification. Since HPLC-quantification is an established and trustworthy method, we can conclude that the colorimetric reaction is not an accurate method to quantify 3,4-DCA for our purposes.

From molecular analysis of the *Variovorax* population a difference in community composition could be detected between the microcosms which received a discontinuous supply of linuron and the microcosms which received no or a single linuron treatment. In fact, under the influence of the discontinuous linuron supply a *Variovorax* population (S) not part of the dominant *Variovorax* populations in soils without linuron supply, seems to have become more dominant. We can hypothesise that this strain is involved in *in situ* linuron degradation.