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Carolina Hoyos-Hernandez, Ariane Bize, T. Bouchez, Laurent Mazéas, Elodie Passeport, et al.. Degradation potential of S-metolachlor in an artificial wetland ecosystem: microcosm assessment. Meeting of SWS/WETPOL and Biogeochemistry symposium, Jul 2011, Prague, Czech Republic. 8 p. hal-01111052

HAL Id: hal-01111052

<https://hal.science/hal-01111052>

Submitted on 29 Jan 2015

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Degradation potential of S-metolachlor in an artificial wetland ecosystem: microcosm assessment

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Abstract. A new method to estimate the biodegradation potential of S-metolachlor, in an artificial wetland ecosystem (AWE) in Bray, France, was developed. For that propose, batch experiments were performed with sediments collected from AWE. The half-life of S-metolachlor was determined under biotic and abiotic conditions (sterile microcosms), aerobic and anoxic conditions and in the presence of different carbon sources (crush plants or acetate). One assay was performed without any carbon source. The results suggest that the S-metolachlor degradation is a biotic phenomenon catalyzed by microorganisms. There is a significant decrease of the half-lives in the biotic microcosm (15.5 days in the case of the aerobic microcosm and 20.5 days in the anoxic) comparing to the sterile microcosms (60.2 days in the case of the aerobic and 45.5 days in the anoxic) ($P < 0.05$). Under aerobic and anoxic conditions the half-lives (12,6 ; 10,8 ; 22,9 et 21,2 ; 14,8 ; 25,7 days) do not show statistical differences ($P < 0.05$). Furthermore, differences were not observed between the assays performed with different carbon sources. Probably due to the absence of cometabolism or simply to the degradation of other carbon sources that could be present in the sediments. The bacterial community dynamics during the experimental period was monitored. The results showed differences between the ARISA profiles during the experiment and also between the different assays. A new band appeared in the ARISA profiles for the microcosms incubated with S-metolachlor, probably corresponding to a new bacterial population, in the presence of crush plants and acetate.

Keywords: Pesticides, Wetland, S-metolachlor; Biodegradation, ARISA, Cometabolism, Half-life

INTRODUCTION

Nowadays, the S-metolachlor is a widely used herbicide to control annual grasses and broad leaf weeds in various crops. One of the reasons of its widespread use in Europe is because metolachlor was retired and replaced by S-metolachlor, which is less persistence in the environment (Pesticide properties DataBase PPDB, website). Today, the information of the transformation rate of S-metolachlor under different conditions is limited while its homologous, metolachlor, has been well-documented for adsorption and degradation in subsoil (Accinelli C et al.; Krutz J et al.). Some of these results suggest that the degradation of this type of pesticide can be strongly influenced by the conditions of oxydo-reduction (Gambrel and Patrick) and others results show that degradation occurred just in a non-sterile surface soil suggesting a microbiological role in the degradation (Accinelli C). However, mainly of these studies are carried out in broth cultures that boost degradation and some of them are developed *in situ* which does not let a simple interpretation, because of the environmental parameters and the lack of differentiation between biotic (plants/microorganisms) and abiotic degradation process (adsorption). The need of information related to S-metolachlor and the limited assessment made until now, justifies the development of a protocol to determine degradation rates and half-lives for S-metolachlor in anoxic and aerobic microcosms, in presence of different substrates which is fundamental in assessing its fate. Finally, the bacterial dynamic was monitored during the incubations under different conditions in order to understand the bacteria evolution.

MATERIALS AND METHODS

Soil

Soil samples were collected from the three parts that compose the artificial wetland ecosystem (AWE) in Bray, France. This site collects all the irrigation waters from 46 hectares of the agrosystem drainage. These waters are charged with 84% of herbicide which S-metolachlor. The soil was collected from the first 5 cm of the AWE. The soil was a luvisol hydro morphed with a layer of natural clay. The soil texture was clay loam sandy (Passport et al.) and its organic matter content was 13.3 %. Collected samples were stored in sterilized plastic bags and transported to the laboratory. Soil samples were passed through a 2-mm sieve to remove roots and rocks. A portion of a soil collected was sterilized by the addition of an HgCl₂ at 1%.

Experimental Setup

In order to test co-metabolic effects in the microcosms, fresh sieved soil (67 g dry weight basis) with 125 ml from AWE was placed in a 1000 ml serum bottle (microcosms) and the volume of a slurry was brought to 250 ml. Additionally, crushed plants (3 g, dw) were added to one treatment named "A"; Acetate (0.1 mg/ml) was added to treatment "B" and to the treatment "C" there was not a complementary source added. The treatments named "Abiotic" with a bacterial inhibitor (HgCl₂) were the controls. Finally, S-metolachlor 97 % purity (2mg/L) was added to each bottle. To analyze the bacterial evolution, a control named "D" was made without S-metolachlor.

To analyze S-metolachlor mineralization, one little bottle with the same proportion in soil and water of the treatments mentioned above was incubated under anoxic conditions with S-metolachlor ring labeled in ¹³carbon (9.6 µg/ml).

The serum bottles were sealed with a septum and some were helium flushed to get the anoxic conditions in the microcosm atmosphere. The bottles were incubated at 20 degrees in light absent. Triplicate bottles were analyzed two-folds a week during 44 days for micro chromatography gas analyses, Gas chromatography mass spectrometry for S-metolachlor in aqueous phase analyses and bacteria ADN soil extraction analyses. Accumulated gas in the head space of the aerobic bottles was released and replaced with air for the duration of the study.

Gas analyses

The gas analyses were done by a gas micro chromatography. Gas composition was analyzed by connecting the bottle to an automatic gas sample injection system (Valco injector and valve for GC, VICI, Switzerland) of a gas chromatograph (IGC CP4900, Varian, USA) equipped with two parallel chromatographic columns coupled to thermal conductivity detectors (TCD). The measurement conditions were the same as in Vigneron et al. (2007). To analyze stable carbon isotopic signature of CH₄ (^δ13C-CH₄) and CO₂ (^δ13C-CO₂), gas samples were collected by a syringe and performed using a Trace GC Ultra (Thermo Electron Corporation, USA) attached to a Delta V plus isotope ratio mass spectrometer (Thermo Electron Corporation, USA) via a Finnigan GC combustion III with an unique combination of micro combustion oxidation furnace. Typical ^δ13C uncertainties were quantified by replicate measurements of a reference CH₄ and CO₂ mixture of known isotopic composition.

Aqueous and solid phase analyses

A syringe was used to remove 2.5 ml during the incubations of slurry from the bottles and the volume was dosed in three Eppendorf lids. After, the lids were centrifuged at 1400 rpm for 10 min in order to separate the aqueous portion from soil portion and to analyze the remaining S-metolachlor in the aqueous phase. The methodology used to detect the S-metolachlor in this phase was gas chromatography-mass spectrometry and this is described in Passport et al. 2010.

For the analyses in soil phase, an estimation of the quantity of S-metolachlor remaining in the sediment was calculated for all the treatments by an external laboratory IPL (NF EN 12393-2 et 3 et XP-X-33012 methodology). This led to analyze the pesticide present in the different treatments at the end of the incubations. In this way, having a general idea of the quantities transformed as a result of biotic or abiotic processes. The solid phase is used also for extract Bacteria ADN. The kit used to extract the ADN Mobio Power soil was chosen after an evaluation of three different kits and methodologies.

Microbiological analysis

The ADN extraction was done with the commercial kit *Mobio PowerSoil* following the producer's recommendations. The bacterial dynamic was monitored during the incubations for all the treatments with the technique (ARISA) Automated Ribosomal Intergenic Spacer Analysis. The Intergenic region spacer (ITS) of bacteria was amplified with - I ITSF (GTCGTAACAAGGTAGCCGTA) and ITSREub (GCFC AAGCATCCACC) primers (Cardinale et al.). For this, 2 µL d'ADN is mixed to Tampon PCR 1X; 0.025 Units/µL of Taq polymerase and 0.2 mM dNTP (kit TaKaRa Ex Taq, TAKARA BIO INC.) was required. After 0.5 µg/ml, T4 gene 32 Protein (BioLabs) and 0.4 µM of each primer was added to get a final volume of 25 µL. The PCR follows this program: 94°C x 5 minutes to 94°C x 1 minute, 54.5°C x 1 minute et 72°C x 2 minutes, and end by 72°C x 10 minutes, 35 cycles. In this way the ADN obtained are used according to kit protocol Agilent DNA 1000 (Agilent technologies Inc.) for label ADN chip detection.

Data analysis

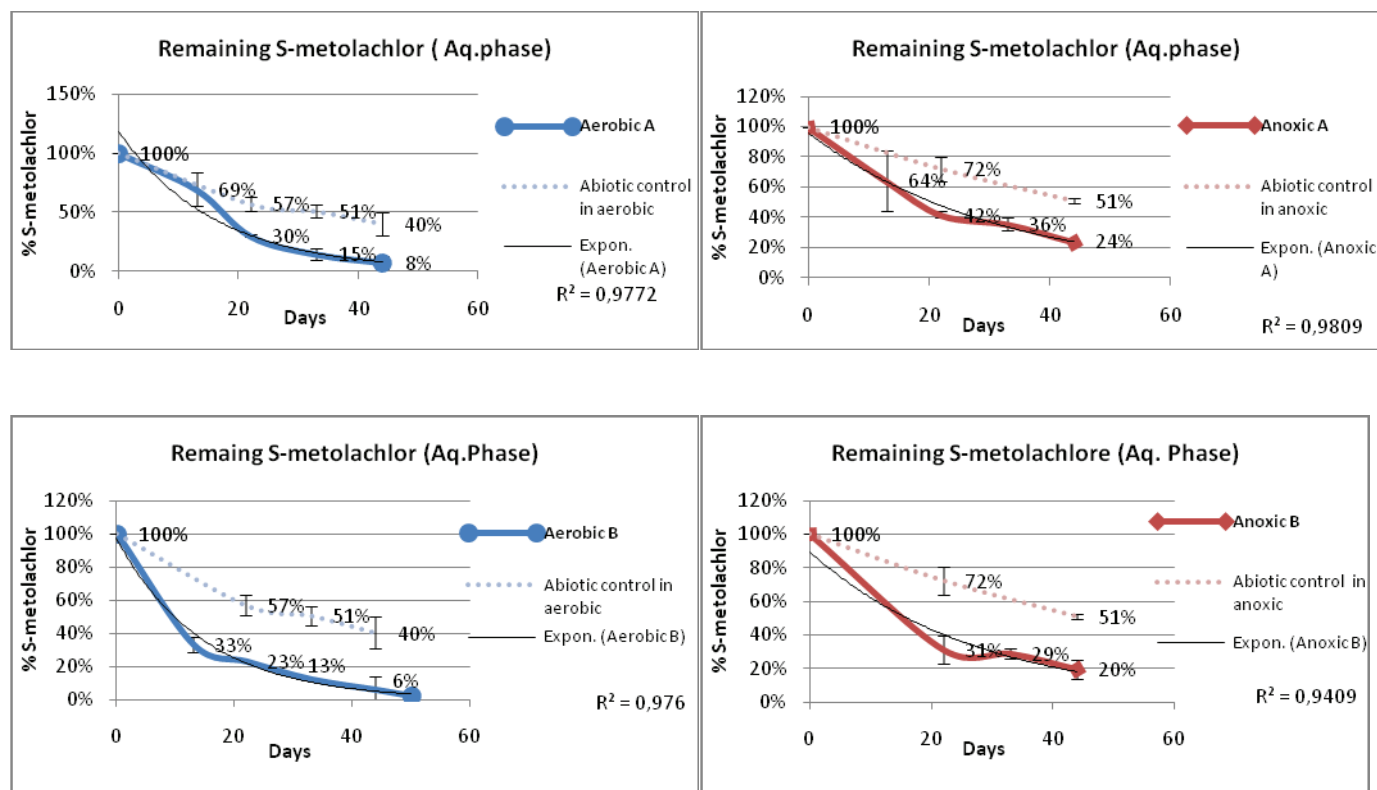
Linear regressions were applied to the data to determine degradation rate constants (k) and half-lives (t_{1/2}) in either natural logarithm of concentration Vs. time plots. Best-fit linear regressions as indicated by the coefficient of determination (r²) were used to determine zero- and first-order reaction rates. Confidence intervals at the 95% probability level were calculated for herbicide half-lives.

RESULTS AND DISCUSSION

The degradation of S-metolachlor in aqueous phase for all treatments **A; B; C** followed first-order kinetics under aerobic and anoxic conditions (Figure 1). The determination coefficients of concentration against time were between 0.94 and 0.98, thus indicating that the curves could fit in exponential first-order kinetics as it has been described for pesticide degradation (Calvet R, Barriso E, Bedor C, Benoit P, M-P Charnay, *Les pesticides dans le sols*. Edition France Agricole 2005). The first effect tested named, biotic and abiotic effects in biodegradation were statistically different (table 1). In the biotic treatments, the mean half-life of S-metolachlor (15.5 aerobic and 20.5 anoxic) was 3 folds lower than that in abiotic experiments, (60.2 and 40.5 days). These results show that there was a significant amount of S-metolachlor that was degraded but also absorbed by sediment as is showed in the abiotics experiments in which there was a decrease of the concentration of S-metolachlor at 44th day of approximately 50%. In the soil that was not sterilized (biotic experiments) there was an increase of almost 30% of the degradation rate that prove that the microorganisms could catalyze the degradation of S-metolachlor (Figure 1). In addition, our results are similar of these obtained for a similar pesticide, Metolachlor, in soil degradation (Accinelli C et al) and these obtained also for Metolachlor degradation tested in bacteria cultures (Miller et al.)

Table1. Mean of half-life for biotic and abiotic treatment.

	Aerobic	Anoxic
Biotic (days)	15.5 ± 6	20.5 ± 4
Abiotic (days)	60.2 ± 2	45.5 ± 5



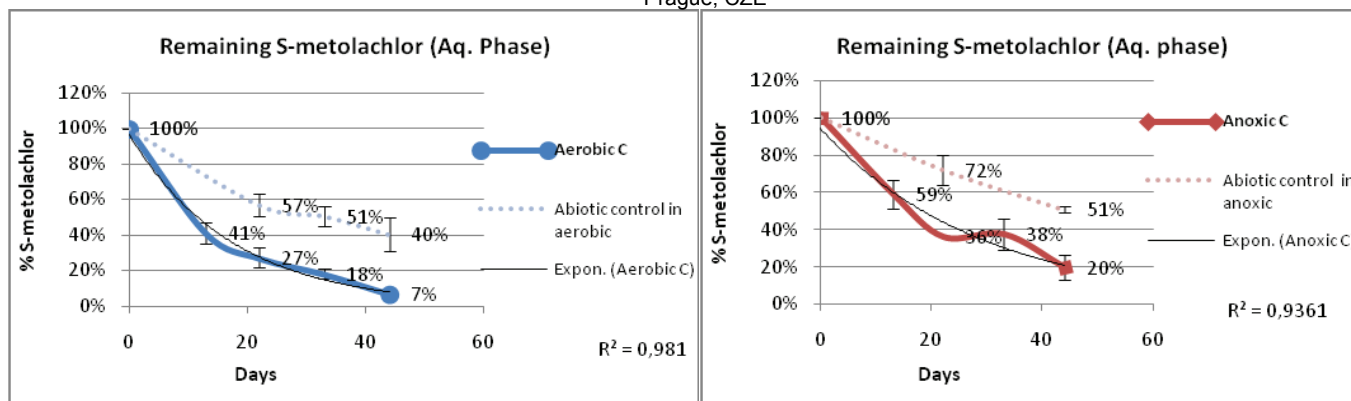


Figure 1. Kinetics graphs under aerobic (right column) and anoxic (left column) treatments. First rank is for A treatments (addition of crushed plants); second rank is for B treatments (addition of acetate) and third rank is for C treatments (without addition of carbon source).

The second effect tested was the addition of different sources of carbon (A crushed plants, B Acetate, C without addition of carbon source) to the microcosms in order to test a co-metabolism related to S-metolachlor degradation. The half-life for these treatments were ranged from 12 to 22 days for aerobic and from 21 to 25 for anoxic conditions (Table 2). Nevertheless, the results did not show statically differences. In fact, the kinetics of degradation show that degradation rates are very similar for the treatments C (without source of Carbon) and B (with acetate) because from 30th to 44th days there is a juxtaposition of curves (Figure 2) that show that there is not a difference between the degradation rates whether an easily-assimilable source of carbon (acetate) was added or not. So, these results suggest that there is not a co-metabolism related to acetate for the S-metolachlor degradation. Nonetheless, the sediments used in our experiment had significant quantity of organic matter (data not shown) that could interact with another co-metabolism for the degradation. That is to say, the presence of an unknown endogenous source of carbon in the sediments could be possibly used by the microorganisms. In consequence, the experiment does not allow to measure co-metabolism effect.

Table 2. Mean of half-life for treatments A B and C under aerobic and anoxic conditions.

	Aerobic	Anoxic
A Crushed plants	12,6±1.6	21,2±1.5
B Acetate	10,8±1	14,8±3
C Without additional carbon	22,9±10	25,7±7.7

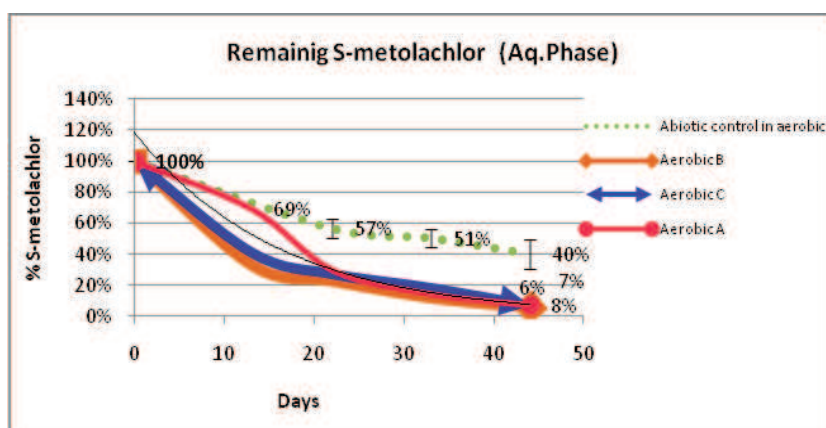


Figure 2. Remaining S-metolachlor in the aqueous phase for treatments in aerobic conditions. A \square (crushed plants) B \diamond (acetate) and C \leftrightarrow (without additional carbon source). (Figure for treatments in anoxic condition are not exposed here).

The third effect tested was: Different incubation conditions.

Some treatments were incubated under anoxic conditions (established as anoxic microcosm because its final electron acceptor is NO_3 SO_4^{2-} present in water from AWE). And the others treatments were incubated in aerobic conditions with a concentration of oxygen maintained in 16 percent during incubations. The kinetics exposed in figure 3 show an augmentation in the degradation rate for treatments in aerobic conditions of 14% compared to anoxic treatments. Nevertheless, the half-life of treatments did not show differences statically significant (Table 3). Our results are not similar to those obtained for its homologue, Metolachlor, which has a rate superior in aerobic than those in anaerobic soils (Miller et al.1997 and Wood et al.1999). Our results could perhaps be attributed to the lack of an effective aeration in the microcosms compared to the one made in nature. In fact, in the nature, the plants let to transport the Oxygen to deeper layers of soil that let to microorganisms have oxygen available to its development in this way increasing the possibility of assimilation of different compounds for the micro aerobic microorganisms (Singh et al, 2004 et 2009).

Table 3. Mean of half-life for treatments in aerobic and anaerobic conditions

	$T_{1/2}$ (days)
Aerobic	15.5 ± 6
Anoxic	20.5 ± 4

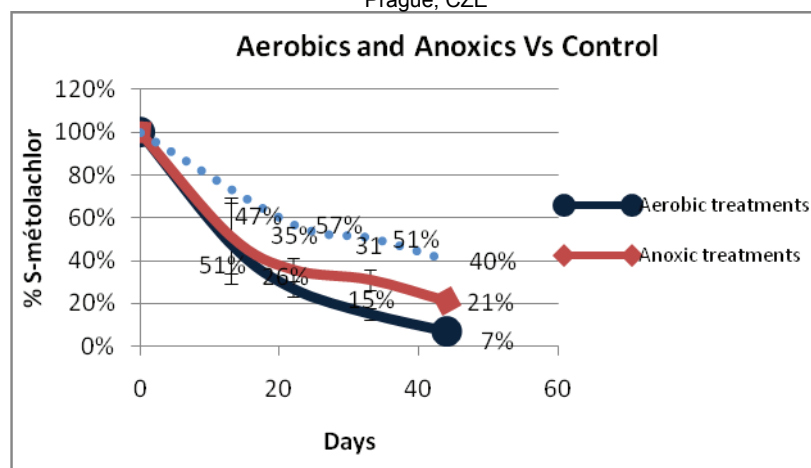


Figure 3. Remaining S-metolachlor in aqueous phase means for treatments in Aerobic \bullet and Anoxic \blacklozenge conditions Vs the abiotic control.

As said before, the microcosms were under different conditions that could change the bacterial structure during the incubations, this changes were analysed with ADN amplification of ITS region. In figure 4, each band of the ARISA profile correspond at least a one bacterial strain while a strain could have several bands. Consequently, the profiles are not easily comparable but it lets to see some changes in the structure of community regarding the different parameters tested (Redox conditions, carbon sources and the controls without S-metolachlor).

These profiles show some differences in the evolution of the bacterial community between the aerobic (right side) and anoxic (left side) conditions and also all those regarding the treatment without S-metolachlor (surrounded by a black rectangle) which was always the same profile.

In those profiles some bands appeared for the treatments with S-metolachlor in aerobic conditions and disappeared at the ends of the incubations. The strongest difference between the profiles in aerobic and anoxic conditions is circled below. This difference shows that at 33th day and 44th the aerobic profiles have two bands which are not as darker as it is in the anoxic profile.

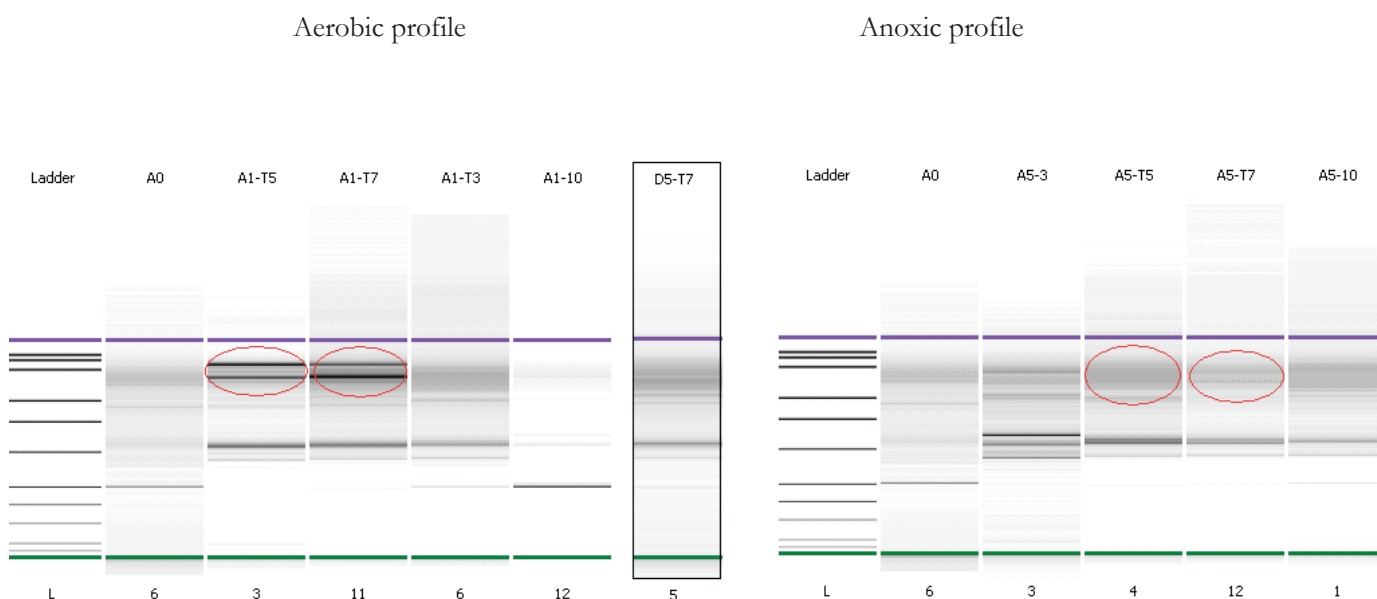


Figure 3. Bacteria diversity profiles for different points of time. Take off day (0), 13th day (T3), 22th day (T5), 33th day (T7), 44th day (T9). At the left: ARISA profiles for aerobic treatments (A1). At the right: ARISA profiles for anoxic treatments. In the middle: ARISA profile for the treatment without S-metolachlor.

The results are also different for whatever Carbone source considered (**A B** or **C**). The different bands between the treatments but often darker correspond to microorganisms who could respond better to substrate added. The aerobic and anoxic are similar between themselves (figure 4). The treatments in anoxic have a base profile stable and conserved between the different treatments at which is added some minority bands that could correspond to microorganisms that could respond better to the substrate. While aerobic profile seems to have more nets bands (surrounded in circles).

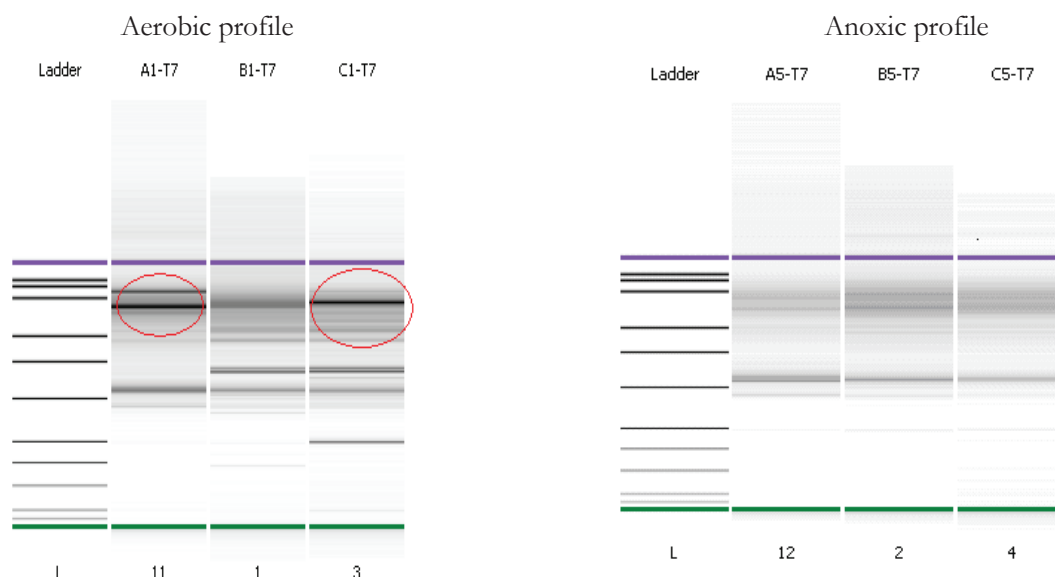


Figure 4. ARISA profile, comparison at 33th (T7) day between different treatments. Treatment “A” for microcosms with crushed plants, “B” for microcosms with acetate, “C” without complementary sourced and “D” microcosms without S-metolachlor.

In anoxic conditions for the ring label molecule, the mineralisation of S-metolachlor it could not be demonstrated by ours experimentations (figure 5) this probably due to the difficult in the ring cleavage or maybe because there were just a transformation to others intermediary metabolites. However, it has been evidenced that its homologous, Metolachlor (label molecule in 14C), is mineralized in 26% in vegetated filters strips (Larry J Krutz et al).

Since Metolachlor seems to be biodegraded, it is interesting to know the conditions for S-metolachlor mineralisation and the microorganisms involved on it. In Further studies SIP technique (stable isotope probing) could be a technique that could offer a powerful new technique for identifying microorganisms that are actively involved in specific metabolic processes under conditions which approach those occurring in situ.

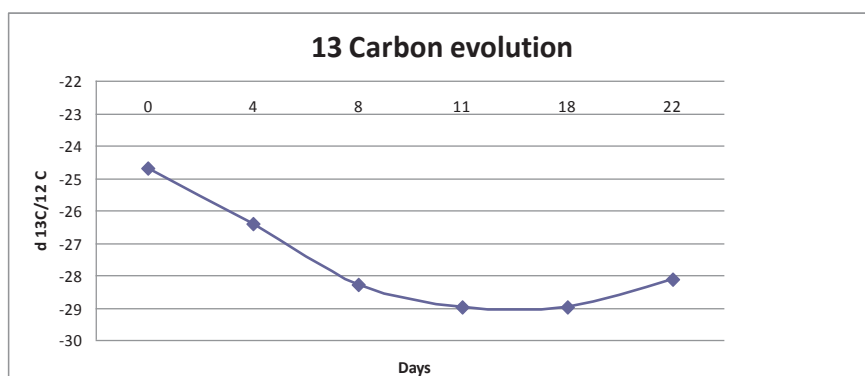


Figure 5. Mineralisation test . Stable carbon isotopic signature of CO₂ evolution for S-metolachlor in time.

Overall, this study suggests that S-metolachlor degradation is influenced by the presence of microorganisms but also to abiotic process as the adsorption. Indeed, our results show that in the final stage of incubations, for the biotic treatments, approximately 64 percent of the total S-metholachlor added corresponds to the remaining portion in the sediments in the aqueous and solid phases (18% and 46% respectively) and the rest, approximately 36%, were transformed by various processes into others products. It would be interesting to monitor its metabolites products . Subsequently the AWE located in Bray France, has a potential in the biotransformation of this pesticide, S-metolachlor. Nonetheless, in further studies is important to analyse the potential of adsorption and desorption as well as the metabolites and sub products of its

transformation in order to see the real quantity that is biodegraded, the environmental impact and if the metabolites are environmentally harmless.

It seems that there is not a co-metabolic process in the presence of acetate or crushed plants in the microcosms or that S-metolachlor is degraded more rapidly in aerobic conditions as Metolachlor does. However, it is important to set up more experiments with a longer period that would show strong differences at the final stages between the treatments.

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