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Estimating the biodegradation of pesticide in soils by monitoring pesticide-degrading gene expression

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Abstract Assessing in situ microbial abilities of soils to degrade pesticides is of great interest giving insight in soil filtering capability, which is a key ecosystem function limiting pollution of groundwater. Quantification of pesticide-degrading gene expression by reverse transcription quantitative PCR (RT-qPCR) was tested as a suitable indicator to monitor pesticide biodegradation performances in soil. RNA extraction protocol was optimized to enhance the yield and quality of RNA recovered from soil samples to perform RT-qPCR assays. As a model, the activity of atrazine-degrading communities was monitored using RT-qPCRs to estimate the level of expression of *atzD* in five agricultural soils showing different atrazine mineralization abilities. Interestingly, the relative abundance of *atzD* mRNA copy numbers was positively correlated to the maximum rate and to the maximal amount of atrazine mineralized. Our findings indicate that the quantification of pesticide-degrading gene expression may be suitable to assess biodegradation performance in soil and monitor natural attenuation of pesticide.

Keywords mRNA · RT-qPCR · Atrazine · Soil RNA extraction · Natural attenuation

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Introduction

The wide use of pesticides in conventional agriculture has increased plant productivity by protecting crops against different pests, but in the meantime it has generated diffuse pollution of chemicals in soil, surface and groundwater. Contamination of natural resources with pesticide residues is of concern for human health since these molecules can reach both drinking water and food resources (Calvet et al. 2005; Younes & Galal-Gorchev 2000). However, the recurrent use of pesticides and their subsequent accumulation in soils led to the selection of microbial communities able to use these xenobiotic compounds as a source of nutrients (Topp 2003). These biodegradation processes can thus reduce the amount of

pesticides in agricultural soils. Soil bacteria can get the ability to degrade pesticides by means of catabolic gene recruitments and catabolic pathway formations through horizontal gene transfer (HGT) of plasmids harboring degradation genes and gene rearrangement mediated by insertion sequences (IS) (Spain et al. 1980; Top & Springael 2003). These degrading-populations are key players of natural attenuation of pesticides in soils. They are responsible for the soil filtering ecosystem service defined in the Millenium Ecosystem Assessment (2005) and for this reason they present ecological and biotechnological interests economically valuable. Detecting the presence of these specific degrading bacterial populations in agricultural soils and unused lands can be useful to assess in situ biodegradation performance and, as consequence, estimate the ability of an ecosystem to naturally attenuate pesticide-contamination and be resilient.

Up to now most of the studies aiming at describing pesticide degradation in the environment were relying on culture-based approaches to enumerate pesticide-degrading bacteria (e.g. Mandelbaum et al. 1995; Radosevich et al. 1995) or on biochemical approaches such as radiorespirometry to estimate potential pesticide-degrading ability (Soulas 1993). The development of molecular approaches relying on direct soil DNA extraction and further analysis by polymerase chain reaction (PCR) have given a new insight into the microbial ecology of pesticide-degrading populations (Martin-Laurent et al. 2004). Indeed, the description of gene sequences coding for pesticide-degrading enzymes allowed different authors to design primer pairs targeting specific pesticide-degrading genes in complex environmental matrices. It has recently been proposed that these genes may constitute sensitive biomarkers reflecting the exposure of a microbial community to pesticides (Bouskill et al. 2007; Ogunseitan et al. 2000). In addition, they may also provide an indication of the pesticide biodegradation performance of a given environment. Different kinds of bacterial indicators have been proposed so far, from the entire bacterial community used as a risk assessment tool to complement contaminant disappearance (White et al. 1998), to the activity of a specific bacterial enzyme used as a biosensor of lead bioavailability (Ogunseitan et al. 2000). Recently the quantification of specific genes such as benzene degraders 16S rRNA gene (Da Silva & Alvarez 2007), catabolic genes involved in chlorobenzene, pyrene, polycyclic aromatic hydrocarbon or 2,4-D mineralization (Baelum et al. 2006; DeBruyn et al. 2007; Dominguez et al. 2008; Park and Crowley 2006) was proposed as a contamination biomarker. However, most of these molecular tools target DNA which gives a distorted view, probably overestimating the pollutant degrading ability due to the quantification of DNA not only present in active bacterial populations but also in dormant and even dead microorganisms (Pietramellara et al. 2009; Wagner 1994). Assessment of the level of expression of these genes should constitute a more reliable and accurate measure of the biodegradation potential since their expression occurs only in metabolically active bacteria. Wagner et al. (2009) recently suggested the use of a *Dehalococcoides* strain gene expression as a functional marker for the natural potential of chlorobenzoate dehalogenation in contaminated sites. Similarly, Baelum et al. (2008) reported the quantification of *tfdA* gene expression as a molecular marker, which should have a deep impact on our understanding of microbial processes responsible for biodegradation of contaminants in the environment. However, this type of approaches remains technically limited since extraction of high quality RNA, which is the prerequisite for gene expression studies, is still difficult to retrieve from soil matrices (Dong et al. 2006; Purdy and Jared 2005). In addition, RNA is rapidly degraded by RNases, which are ubiquitous enzymes naturally produced by the indigenous soil microorganisms and thereby compromising the recovery of high quality RNA from soil samples. In this context, the objective of our study was to study the suitability of quantifying the expression of pesticide-degrading genes as an indicator of microbial biodegradation in five agricultural soils showing contrasting physico-chemical properties. As a model, the herbicide atrazine, widely used to control broadleaf weeds development in corn crops, was used in our study.

Different quantitative PCR assays were previously reported to measure the abundance of *atz* or *trz* sequences coding for atrazine-degrading enzymes using environmental DNA as template (Thompson et al. 2010). These assays have been successfully used for monitoring the abundance of atrazine-degrading genetic potential in environmental samples where it was found to be positively correlated to atrazine concentration measured in lake water and sediments (Sherchan and Bachoon 2011). However, some studies showed discordances between abundances of atrazine-degrading genetic potential and atrazine-mineralizing activity monitored in different soil types (Martin-Laurent et al. 2004). These discrepancies might be due to the

weakness of the correlation of the size of the atrazine-degrading genetic potential and the estimation of the activity resulting from its expression, which is regulated at several levels in bacterial cells and placed under the influence of environmental variations. In this context, tracking the abundance of *atz* RNA transcripts in environmental samples would represent a step forward toward the atrazine-degrading activity. Up to now, the *atz* full pathway expression has only been reported on pure strain cultivated under control conditions (Devers et al. 2004). Keeping in mind that the upper catabolic pathway of atrazine can rely on both *atzABC* and *trzN* genes, the last tending to rapidly expend according to recent biogeographic study (Arbeli and Fuentes 2010) and to the recent isolation of atrazine-degrading strains harboring *trzN* genes (El Sebaï et al. 2011; Udiković-Kolić et al. 2011), we have decided to target the *atzD* gene as a molecular marker to estimate the performances of microbial communities mineralizing atrazine. Moreover, we recently reported that *atzA* mRNA was not easily detected in different agricultural soils (Monard et al. 2010). *atzD* is the first gene of the lower pathway transforming cyanuric acid to CO_2 and NH_4. It is highly conserved among atrazine-degrading populations and codes for AtzD, an enzyme opening the *s*-triazine ring of cyanuric acid, a key intermediary metabolite of atrazine (de Souza et al. 1996, 1998a; Martinez et al. 2001). As part of the *atzDEF* operon, this gene is placed under the regulation of the *atzR* transcription factor (Garcia-Gonzalez et al. 2005). *atzD* expression is up-regulated by cyanuric acid and should thus be closely linked to the rate of cyanuric acid degradation and thus to atrazine mineralization. In the present study, RNA extraction and purification techniques were optimized to obtain a high yield of high quality RNA from five different agricultural soils. Then, the 16S rRNA and *atzD* gene expressions were quantified by reverse transcription-quantitative PCR (RT-qPCR) from soil RNA. The relative abundance of *atzD* mRNAs was correlated to the ability of soil microbial community to mineralize atrazine.

Materials and methods

Soil samples

Soil samples of cropped plots were collected in autumn 2007 from the surface-layer (horizon 0–20 cm) of five different agricultural sites located in France: Epoisses (Côte d'Or), Vezin (Ille et Vilaine), La Côte Saint André (LCSA) (Isère), Montrond (Isère) and Kerguehennec (Morbihan). These soils were chosen, not only for their similar cropping history, but also for their different particle size distributions, pH and organic matter contents (Table 1). For more than a decade, these soils were cropped with maize/wheat rotations and as a consequence, they have for a long time been regularly exposed to atrazine (one treatment every 2 years at 1.5 kg ha^{-1}). Soils were sieved at 4 mm and kept at 4 °C until use. Aliquots of soils for RNA extraction were frozen in liquid nitrogen and kept at −80 °C until use.

RNA extraction

Based on the cell lysis procedure for RNA extraction described by Courty et al. (2008) we developed a RNA extraction protocol to obtain high quality and high quantity of RNA from 250 mg of soil. The optimization of the cell lysis consisted in: (i) adjusting the pH to 6.6 by using phosphate buffer (0.1 M, pH 6.6) to prepare the lysis solution and phenol solution (Fluka Sigma-Aldrich) saturated with 0.1 M phosphate buffer (pH 6.6) and (ii) using bead beating of the soil samples instead of mortar grinding. The optimized cell lysis was thus as followed: 250 mg fresh soil, 0.5 g glass beads (106 µm; Sigma-Aldrich), 200 µl phosphate buffer (0.1 M, pH 6.6), 33.5 µl 20 % sodium dodecyl sulfate, 170 µl 3 % diatomaceous earth (Sigma-Aldrich), 800 µl of phenol 2-β-mercaptoethanol solution (pH 6.6, 0.2 % vol/vol) were processed in a bead beater for 3 min at 1,800 rpm with a freezing step in liquid nitrogen each minute. After centrifugation at 15,000×*g* for 10 min at 4 °C, a phase separation step was added by mixing the supernatants with an equal volume of phenol–chloroform-isoamyl alcohol (25:24:1, pH 6.6) and the samples were centrifuged at 20,000×*g* for 15 min at 4 °C. The phenol was removed from the aqueous phase by mixing with an equal volume of chloroform-isoamyl alcohol (24:1) followed by centrifugation at 20,000×*g* for 15 min at 4 °C. Total nucleic acids were then precipitated from the aqueous layer with 0.1 volume of 8 M LiCl and 2.5 volumes of cold ethanol and incubated for 2 h at −80 °C. The pellets obtained after centrifugation at 20,000×*g* for 30 min at 4 °C were washed with cold ethanol (75 %), dried at room temperature, and

dissolved in 100 µl of ultrapure DNase- and RNase-free water. RNAs were separated from DNAs using Trizol (Chomczynski 1993; Chomczynski and Sacchi 1987; Sambrook and Russell 2001). Thus according to the manufacturer's instructions, 1 ml of TriReagent (Sigma-Aldrich) was added to the previously isolated nucleic acids. The samples were vortexed and incubated for 5 min at room temperature. Chloroform (200 µl) was added and the aqueous phase was collected after centrifugation at 20,000×g for 15 min at 4 °C. Total RNAs were precipitated by the addition of 500 µl isopropanol and incubation at room temperature for 10 min. After centrifugation at 20,000×*g* for 10 min at 4 °C, the pellets were washed with ethanol (75 %), dried at room temperature and dissolved in 50 µl of ultrapure DNase- and RNase-free water. RNA samples were treated with RNase-free DNase I in the presence of RNasin® Plus RNase inhibitor (Promega) according to the manufacturer recommendations and an aliquot of 4 µl was used as template in a PCR reaction to control the lack of amplification. This procedure was used to extract RNA from 3 replicates of each of the five different soils. RNA extracts were stored at −80 °C until their use.

Evaluation of RNA quality and quantity

RNA was quantified by spectrophotometry at 260 nm and RNA purity was estimated by calculating the 260/280 and 260/230 absorbance ratios (NanoDrop® ND-1000, NanoDrop Technologies). RNA quality was assessed by microfluidic electrophoresis using the Experion Automated Electrophoresis System and RNA HighSens lab-chips (Bio-Rad Laboratories).

Microbial gene expression

RT-qPCR reactions were performed to quantify the 16S rRNA and *atzD* gene expression in the total RNA extracts from three replicates of each of the 5 different soils ($n_{tot} = 15$). Reactions were performed as described by Devers et al. (2004) with 125 ng of RNA. The primers 515R 5′-TTA CCG CGG CTG CTG GCA C-3′ (Xia et al. 2000) and Dr 5′-GGG TCT CGA GGT TTG ATT G-3′ (Devers et al. 2004) were used for the reverse transcription of 16S rRNA and *atzD* mRNA respectively. Ten units of AMV-RT and 200 units of MMLV-RT (Promega) were used as reverse transcriptases. The quantitative PCRs were carried out in an ABI Prism 7900HT (Applied Biosystems) apparatus with SYBR green PCR Master Mix (Absolute Green Rox ABgene). The 25 µl reaction mixtures contained 10 µl of QPCR SYBR master mix, 0.3 µM of the forward primer (341f 5′-CCT ACG GGA GGC AGC AG-3′ (Muyzer et al. 1993) or Df 5′-TCC CAC CTG ACA TCA CAA AC-3′ (Devers et al. 2004) for the amplification of 16S rRNA and *atzD* mRNA, respectively), 0.3 µM of the reverse primer (515r or Dr), 5 µl of cDNA template, 1 µl of T4 gp 32 (QBiogene) and 5 µl of ultrapure water. The thermal cycling conditions for the 16S rRNA sequence amplification consisted of an initial step of 2 min at 50 °C and 10 min at 95 °C followed by 35 cycles at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s and 81 °C for 15 s. The last cycle consisting of 15 s at 95 °C, 15 s at 60 °C and 15 s at 95 °C allowed the production of dissociation curves. Identical thermal cycling conditions were used to quantify the *atzD* mRNA except for the initial step consisting of 10 min at 95 °C followed by 40 cycles instead of 35.

The calibration curves were as follows (supplementary figure):

$$Ct = -3.3 \times \log(\text{number of 16S rRNA sequences}) + 38.6 \; (R^2 = 0.99)$$

$$Ct = -3.0 \times \log(\text{number of } atzD \text{ sequences}) + 35.4 \; (R^2 = 0.99)$$

Atrazine mineralization

Atrazine mineralization was measured by radiorespirometric analyses estimating the amount of $^{14}CO_2$ evolved from ^{14}C-ring labeled atrazine as described by Soulas (1993). The kinetics of mineralization in the Epoisses, Vezin, LCSA, Montrond and Kerguehennec soils were performed using miniaturized experimental systems (Gonod et al. 2006). Soil samples (0.5 g) were moistened to 23 % humidity, treated with 128 Bq of ^{14}C-labeled atrazine (910 MBq mmol^{-1}, 99 % purity), on the basis of 1.5 mg per kg of dry soil and incubated in darkness for 68 days. The $^{14}CO_2$ output was precipitated on Whatman paper soaked in H_2BaO_3 and after 2 h at 80 °C the membranes were exposed to ^{14}C sensitive screens which were then scanned using phosphor imager (Storm®, Molecular Dynamics). The data were then analyzed with the ImageQuant program (Molecular Dynamics). Atrazine degradation capacity was expressed as the percentage of ^{14}C-CO_2 in relation

to the initial quantity of ^{14}C-atrazine. The initial radioactivity was determined after quantification of 25 µl of ^{14}C-atrazine stock solution by liquid scintillation counting (Packard Tricarb 1900). The modified Gompertz growth model ($y = Ae^{-e^{\left(\frac{\mu_m \times e}{A}(\lambda - t)+1\right)}}$; $P < 0.05$) was used for modeling atrazine degradation kinetics. Three parameters were determined: A, the plateau or maximum amount of atrazine mineralized (% of ^{14}C-atrazine initially added); μ_m, the maximum mineralization rate constant (% of ^{14}C-atrazine initially added per day); and λ, the lag time (day).

Results

Extraction of RNA from different soils

The optimized protocol of direct RNA extraction was tested on five different agricultural soils and was compared to the original protocol described by Courty et al. (2008). The pH of the cell lysis buffer was adjusted to 6.6 after preliminary assays conducted at three different pH values (8.0, 6.6 and 5.0) and showing a better RNA yield at this pH (data not shown). This impact of the pH of the cell lysis buffer on RNA recovery from soil is in accordance with previous report identifying pH as a key parameter affecting both RNA precipitation and stability (Ameziane et al. 2006). The quality of the RNA extracted from the five different soils with the optimized method was attested by the observation of clear cut SSU rRNA and LSU rRNA bands. On the contrary, RNA extracted using the original protocol showed a fuzzy profile, which presented additional bands (Fig. 1). The spectrophotometric quantifications of RNA and those obtained by microfluidic electrophoreses were similar (data not shown).

The highest RNA extraction yield was obtained from the soil of Montrond (5.6 µg RNA g^{-1} dry soil) while the lowest was obtained from the Epoisses soil (1.6 µg RNA g^{-1} dry soil) (Table 1). The yield of RNA extracted from the different soils was significantly positively correlated to the sand content of the soils ($R^2 = 0.57$, $P < 0.01$) and, to a lesser extent negatively correlated to the soil pH ($R^2 = 0.26$, $P = 0.05$) (Table 1).

Gene expression quantification

The 16S rRNA quantified from RNA extracted from the five soils ranged from 1.5×10^{11} copy number per

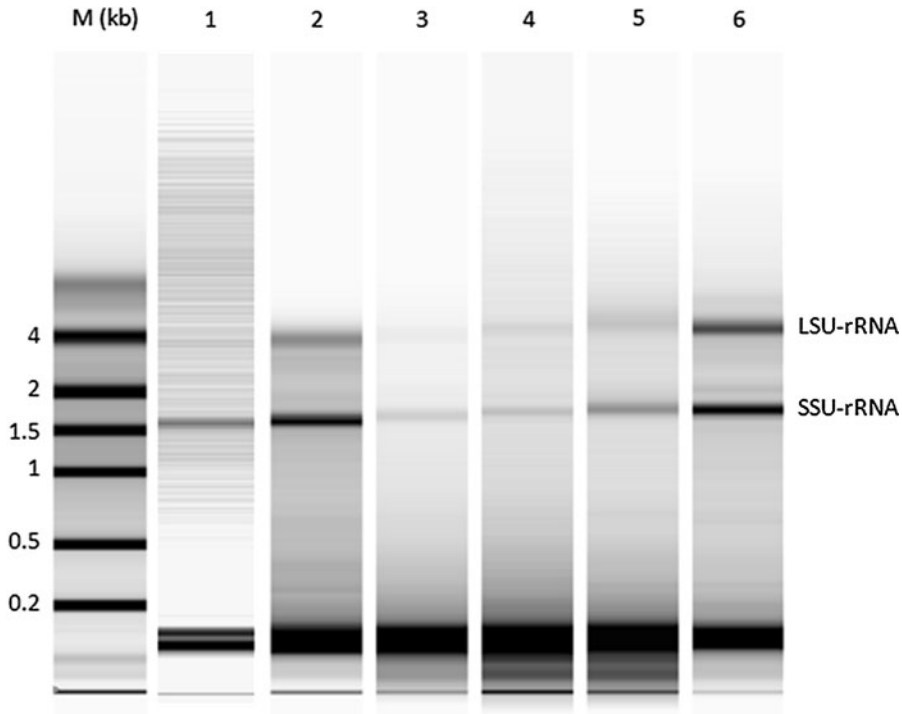

Fig. 1 Microfluidic electrophoresis of total RNA (adjusted at 2 ng µl^{-1}) extracted by using the method described by Courty et al. (2008) on the Epoisses soil (*1*) and the optimized protocol on the Epoisses (*2*), Vezin (*3*), LCSA (*4*), Kerguehennec (*5*) and Montrond soils (*6*). *M* Ladder

Table 1 Soil physico-chemical characteristics and RNA extraction yields (mean ± SD, $n = 3$)

Soil	Particle size distribution (%)			pH	Organic matter (%)	C/N	Moisture (%)	μg of RNA per g of dry soil
	Clay	Loam	Sand					
Epoisses	40	49	11	8	3.9	10.2	24	1.6 ± 0.4
Vezin	14	68	17	6.4	2.3	9.4	20	2.9 ± 0.1
LCSA[a]	14	43	43	7	2.3	10.3	19	4.5 ± 0.0
Kerguehennec	18	51	31	5.9	5	10.5	22	4.7 ± 0.7
Montrond	24	32	44	6.7	6.1	9.6	33	5.6 ± 0.0
Least square regression (R^2)	0.21	0.22	0.57	0.26	0.16	0	0.1	
P^2	0.09	0.08	<0.01	0.05	0.14	0.99	0.24	

Least square regressions of the RNA yield against each physico-chemical property and corresponding P values are presented

[a] LCSA La Côte Saint André

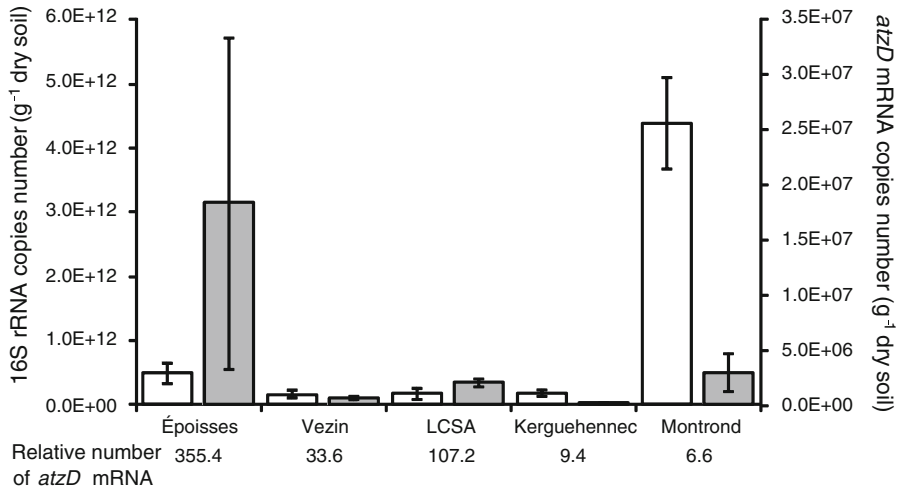

Fig. 2 16S rRNA (*white*) and *atzD* mRNA (*grey*) copy number per gram of dry soil in the Epoisses, Vezin, LCSA, Kerguehennec and Montrond soils. *atzD* mRNA abundances are given in number of *atzD* mRNA copy per 10^7 16S rRNA copy number

gram of dry soil for the soil of Vezin to 4.4×10^{12} copy number per gram of dry soil for the soil of Montrond (Fig. 2). The abundance of active bacteria was positively correlated to the soil moisture ($R^2 = 0.92$, $P = 0.01$). The lowest expression of *atzD* gene was recorded for the soil from Kerguehennec (1.9×10^5 mRNA copy number per gram of dry soil) while the highest was found in the soil of Epoisses (1.8×10^7 mRNA copy number per gram of dry soil) (Fig. 2). The expression of *atzD* gene was normalized against 16S rRNA abundance. The relative number of *atzD* mRNA greatly varied between the different soils: from 6.6 *atzD* mRNA per 10^7 16S rRNA in the Montrond soil to 355.4 *atzD* mRNA per 10^7 16S rRNA in the Epoisses soil (Fig. 2). It was shown to be significantly correlated to the soil pH ($R^2 = 0.85$, $P = 0.03$).

Atrazine mineralization

The five soils presented different atrazine mineralization abilities (Fig. 3) and they could be clustered into three groups. The first group, formed by the Epoisses soil showed a first order kinetics typical for soil microbial communities adapted to pesticide mineralization, without any distinctive lag phase (0.16 days according to the modified Gompertz growth model). The second group formed by the LCSA and Vezin soils, also showed a first order kinetics but with a lag phase ranging from 10 to 13 days. The third group formed by Kerguehennec and Montrond soils showed zero order kinetics typical from co-metabolic biodegradation and/or abiotic degradation. Similar grouping could be done using kinetics parameters resulting from modeling with the modified Gompertz growth model.

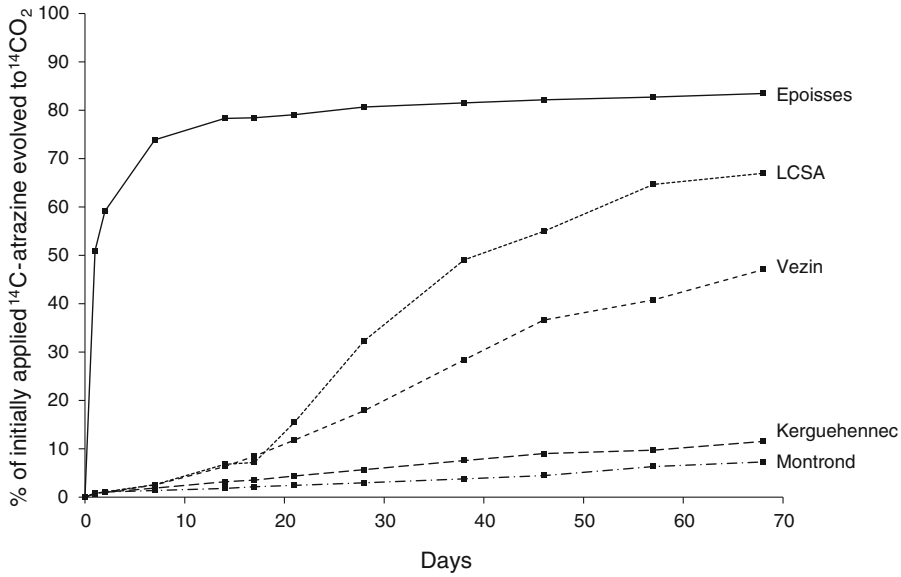

Fig. 3 Atrazine mineralization kinetics recorded in the soils of Epoisses, Vezin, LCSA, Kerguehennec and Montrond expressed as percent of initially applied ^{14}C-atrazine evolved to $^{14}CO_2$

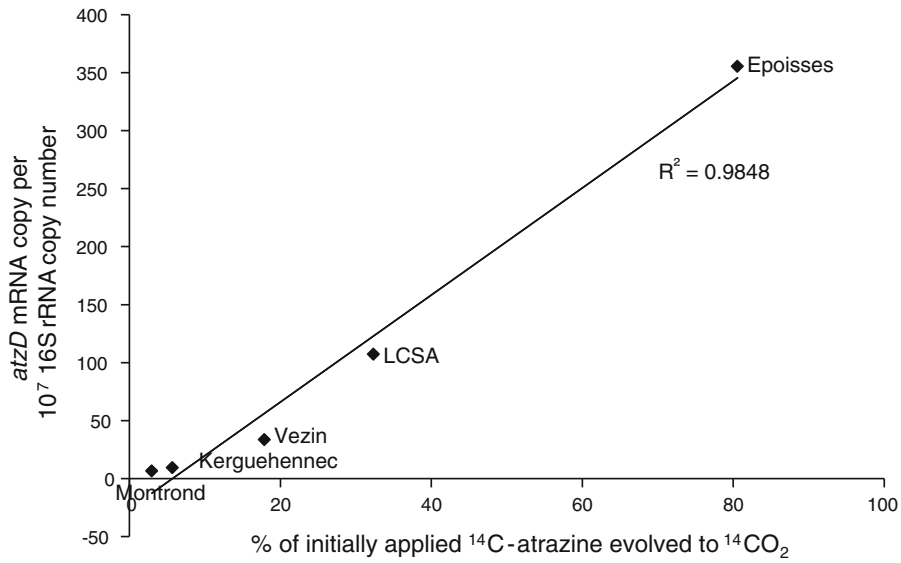

Fig. 4 Correlation between the number of *atzD* mRNA copy per 10^7 16S rRNA copy number and the maximal percentage of $^{14}CO_2$ evolved from ^{14}C-atrazine initially applied

The maximum rate of atrazine mineralization (μ_m) was high in the Epoisses soil (62 days^{-1}), medium in the LCSA and Vezin soils (2 and 1 day^{-1}, respectively), and low for Kerguehennec and Montrond soils (below 1 day^{-1}). Interestingly a positive correlation between the relative number of *atzD* mRNA and the atrazine mineralization ability (percent of substrate mineralized) estimated in the five soils was observed until 45 days of incubation ($R^2 = 0.98$, $P = 0.001$; Fig. 4). The maximum mineralization rate, μ_m, was the only parameter determined using the modified Gompertz growth model significantly related to the relative number of *atzD* mRNA ($R^2 = 0.94$, $P < 0.01$; data not shown).

Discussion

The recent advances in molecular biology allowed the development of new tools based on genomics to monitor functional microbial communities involved in different soil ecosystemic services including natural

attenuation of pesticides accounting for 'the filter service' of soil (e.g. Kazy et al. 2010). In a bioremediation context, microbial molecular biomarkers have been proposed using either DNA or RNA and focusing on 16S rRNA or catabolic genes involved in pollutant degradation. For example, Da Silva and Alvarez (2007) developed specific 16S rRNA primers to quantify the benzene degrading bacteria and assess the anaerobic benzene degradation potential in aquifers. But, as shown by Kazy et al. (2010) and Peng et al. (2010), the 16S rRNA gene is not always related to the biodegradation function of interest even if it is specific to bacterial groups prevalent when the degradation occurs. Indeed, the validity of using the 16S rRNA gene as an indicator of biodegradation performance relies on its congruence with the functional phylogeny. This strong hypothesis is unlikely for pesticide degraders since the pesticide-catabolic genes are known to be hosted in plasmids readily exchangeable between bacterial populations and often mobilisable by IS sequences (de Souza et al. 1998b; Topp 2003), increasing the diversity of the bacterial phylotypes supporting the function (Monard et al. 2011), and thus not congruent with 16S rRNA phylogeny. In order to assess the in situ biodegradation performance using molecular tools targeting functional microbial communities, it is thus better to focus on specific genes coding for catabolic enzymes as suggested by DeBruyn et al. (2007) who observed that mycobacterial catabolic genes were functionally associated to pyrene mineralization. Quantification of catabolic genes reveals the biodegradation genetic potential of a given soil (Monard et al. 2008), but it does not guarantee the expression and the achievement of the function because it assesses the entire microbial communities regardless of the activity of individual components (Prosser 2002). In contrast, quantification of mRNA which is transcribed in response to exposure to the pollutant is more representative of the physiological state of microbial populations involved in the biodegradation process (Kong and Nakatsu 2010).

In order to evaluate the suitability of mRNA for monitoring biodegradation performance, high quality RNA is needed, which is difficult to retrieve from complex environmental samples such as soils. The soil RNA extraction protocol presented herein allowed the recovery of high quality RNA and was shown to be suitable for extracting RNA from five agricultural soils showing contrasting soil physico-chemical characteristics. The significant impact of the soil sand content observed on the RNA yield could be explained by the mechanical impact of sand during the cell lysis step. Such as the glass beads and the diatomaceous earth used in our protocol, sand can also be used as a mechanical agent to improve the disruption of cell walls during bead-beating or grinding and so can increase the final RNA recovery (e.g. Hurt et al. 2001). In addition, the lowest yield of RNA obtained in the Epoisses soil particularly rich in clay particles suggests that clay amount could be inversely correlated to RNA extraction by adsorbing nucleic acids.

The suitability of our RNA extraction protocol for gene expression studies was further confirmed by successful RT-qPCR assays performed to monitor active bacteria (16S rRNA) and atrazine-degrading bacterial populations (*atzD* mRNA) in five different soils. The application of gene expression quantification to assess the pesticide-biodegradation performance in soils was addressed by using the herbicide atrazine as a model. Indeed, it has been extensively and widely used to prevent the growth of weeds in corn crop over the last 50 years and although its use has been banned in year 2003 in France, it is still often detected in water resources (Commissariat général au développement 2011). As a consequence, its biodegradation in soil has been extensively studied leading to the identification of the different *atz* and *trz* catabolic genes coding enzymes involved in its degradation (de Souza et al. 1998a; Martinez et al. 2001). The *atzD* gene was chosen as a target gene to assess atrazine biodegradation performance because (i) it encodes the *s*-triazine ring cleavage, a key step in atrazine degradation, close to the final mineralization function and (ii) the *atzDEF* operon is regulated by AtzR (Garcia-Gonzalez et al. 2005), a transcription factor belonging to the *LysR* family (Martinez et al. 2001). Recently, Helbling et al. (2011) reported that the level of expression of *atzA*, the first gene involved in atrazine biodegradation (de Souza et al. 1996), was positively correlated to atrazine-degrading activity in bioreactors amended with an atrazine chlorohydrolase-recombinant strain. However, *atzA* being constitutively expressed (Devers et al. 2004), the quantification of its expression would rather reflect the amount of active atrazine-degraders than effective biodegradation. One could hypothesize that this is not a problem in optimized conditions (Helbling et al. 2011), but it may not be suitable when applied to

complex systems such as soils where several atrazine-degrading upper pathways (*atzABC* vs *trzNBC*) coexist (Devers et al. 2007). In addition, we recently shown that the expression of *atzA* in soil was not always detected even when atrazine biodegradation occurred (Monard et al. 2010).

In the present study we reported a significant positive correlation between the relative amount of *atzD* and the atrazine mineralization kinetics for the five arable soils tested. Successful quantification of *atzD* gene expression from total RNA extracted from soils differing in their physico-chemical properties and in their performance in atrazine biodegradation proves that this method can broadly be applied to assess natural attenuation of atrazine. It is noteworthy that even if the soils have not been treated with atrazine since the ban of this herbicide in France in 2003, three over five (Epoisses, LCSA and Vezin) showed kinetics of mineralization of the first order, typical for enhanced biodegradation. As previously observed by Cheyns et al. (2012), these results point out the long-term survival of atrazine-degrading bacteria after application of the pesticide and their ability to recolonize the soil after new atrazine treatments. In the two other soils (i.e. Montrond and Kerguehennec) one could hypothesize either that atrazine-degrading communities were not selected when atrazine was regularly applied or that if selected under atrazine selection pressure they perished in the absence of atrazine.

As already observed, the mineralization of atrazine by indigenous microbial communities was highly dependent on soil pH, with lower atrazine mineralization recorded in acidic soils (Houot et al. 2000; Monard et al. 2010). Interestingly we reported for the first time that the expression of *atzD* was also dependent on soil pH, with higher *atzD* mRNA recorded in basic soils. Soil pH, by acting on both biological and physico-chemical components of the soils seems to be one of the main factors controlling microbial degraders activity and pesticide bioavailability (Bending et al. 2006).

Assessing the in situ performance of pesticide biodegradation in soil is of great interest since soil is a recharge zone of aquifer having filtering capabilities that can limit pollution of groundwater. The development and use of functional gene microarrays have already been proposed to evaluate microbial potential of pesticide degradation in rural aquifer (He et al. 2007; Liebich et al. 2009). However, by being only qualitative, this tool estimates the biodegradation potential presents in an environment without monitoring its level of expression. By using atrazine as a model, we demonstrated that the expression level of catabolic genes was a sensitive and accurate indicator to monitor the biodegradation performance in agricultural soil and to assess natural attenuation. We can speculate that this approach could be applied to other pesticides for which degradation genes have been identified, such as diuron (*puhA*), linuron (*libA*), carbofuran (*mcd*), organophosphate (*opd*) or 2,4-dichlorophenoxyacetic and 4-chloro-2-methylphenoxyacetic acids (2,4-D and MCPA) (*tfdA*)… (Bers et al. 2012; Fukumori and Hausinger 1993; McDaniel et al. 1988; Parekh et al. 1995; Turnbull et al. 2001). Moreover, a high degree of correlation between expression of *tfdA* and the rates of mineralization of 2,4-D and MCPA in soil have already been observed (Baelum et al. 2008), thereby reinforcing our results. The use of molecular methods to assess the biodegradation performance of soil is of great interest for natural attenuation monitoring and the quantification of specific catabolic gene expression constitutes promising sensitive indicators of in situ biodegradation activity.

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