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# Metabolic characterisation of fifteen atrazine-degrading microbial communities

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**Fifteen atrazine-degrading microbial communities obtained from different sources were able to degrade atrazine in a liquid mineral medium as the main organic substrate at high rates (atrazine half-lives ranging from 20 to 164 h). Hydroxyatrazine was the sole metabolite detected. This metabolite was always transient but its maximum level varied from 4 to 67% of the parent compound. Communities originating from subsurface sediments degraded atrazine at similar rates (half-lives between 56 and 62 h). A Biolog characterisation revealed a wide diversity of substrate utilisation by the communities originating either from the surface or the subsurface environments. Twenty-four Biolog carbon sources were degraded by the fifteen communities. A multiple regression analysis established a statistically significant relationship between the atrazine DT50 values of thirteen communities and their responses to four Biolog carbon sources.**

**Keywords:** atrazine; Biolog; community; biodegradation

## Introduction

Atrazine (ATR), 2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine, is a widely used herbicide. Its moderate properties of persistence and adsorption have ensured its success as both a total and a selective herbicide, mainly for maize crops in Europe and the United States. However, these same properties are now leading to extensive contamination of surface water [32,34,35] as well as aquifers [4,40]. Thus, the interest in its elimination from water has been growing. Various methods such as ozonation [19,27] and/or adsorption on activated carbon [26] have been suggested to clean atrazine-contaminated water. Amongst these approaches, biodegradation is a promising alternative since it could allow complete elimination of the contaminant. It is only recently that atrazine biodegradation and mineralization of the *s*-triazine ring has been reported in surface and subsurface soils [5,37,39,41] by bacterial communities [1,3,24,30,33,38] and by microbial isolates [23,29,43]. Surprisingly, all these reports have confirmed the possibility of biological dechlorination, first reported by Behki and Khan [6], whereas in the past, hydroxyatrazine (HA) had been attributed exclusively to abiotic processes [2,6,21]. As a consequence, the main transient metabolite reported in these most recent studies is HA and not dealkylated compounds as reported elsewhere [7,8,25,31].

In 1991, Garland and Mills [16] introduced the use of Biolog microplates to characterise and classify heterotrophic microbial communities. According to these authors, the main advantages of this method are that it allows a community-level approach, eliminating some limitations of the isolate-based methods, and that it is based on the functional capacities of the bacterial community that may be of ecological interest. Even though the basal medium used in the Biolog plates has been shown to be somewhat selective [18], the direct incubation of whole environmental samples allows keeping the community as close as possible to its initial state. Recent studies have shown the utility of Biolog microplates to characterise entire communities. This method has been used successfully to study some rhizospheric communities [15,17], the evolution of bacterial consortia within cultivated [10] or contaminated soils [22,42].

In this study, the biodegradation of atrazine in liquid media by fifteen microbial communities was studied. The Biolog method was used to further characterise the metabolic properties of these communities. The majority of them were taken from surface soils of fields intensively cultivated with maize. Several others originated from other types of surface soils or from subsurface soils. The consortia were obtained from four different countries, a majority among them from Belgium [28]. What all these ecosystems had in common was that they had received repeated *s*-triazine (atrazine and simazine) treatments and that they have exhibited high atrazine dissipation rates. The aim of this study is to illustrate the similarities as well as the diversity of the microbial communities able to rapidly degrade atrazine.

## Materials and methods

### *Origins of the fifteen microbial communities*

The fifteen microbial communities and their origins are presented in Table 1. With the exception of COM14 [33], all the communities were obtained from soils initially exhibiting half-life values for atrazine (ATR) lower than 15 days at 25°C [9,13,28,39]. Considering that the half-life of atrazine in soils is generally estimated to be between 6–10 weeks [20], these soils can be considered as highly active with regard to atrazine degradation. This feature may be attributed in the case of the maize fields to previous annual

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**Table 1** Origins of the different atrazine-degrading microbial communities

| Community | Country (Place) | Type | Reference |
|---|---|---|---|
| COM1 | Belgium (Corroy) | Maize field (soil surface) | [28] |
| COM2 | Belgium (Pont) | Maize field (soil surface) | [28] |
| COM3 | Belgium (Wavre) | Maize field (soil surface) | [28] |
| COM4 | Belgium (Wagnelée) | Maize field (soil surface) | [28] |
| COM5 | Belgium (Fouron) | Maize field (soil surface) | [28] |
| COM6 | Ireland (–) | Pedestrian way (soil surface) | [13] |
| COM7 | Ireland (–) | Tennis court (soil surface) | [13] |
| COM8 | Hungary (Debrecen) | Maize field (soil surface) | [9] |
| COM9 | Belgium (Sart-Dames) | Maize field (soil surface) | [28] |
| COM10 | Belgium (Korbeek 1) | Maize field (soil surface) | [28] |
| COM11 | Belgium (Korbeek 2) | Maize field (soil subsurface) | [39] |
| COM12 | Belgium (Korbeek 3) | Maize field (aquifer) | [39] |
| COM13 | Belgium (Louvain-1-N) | Maize field (soil subsurface) | [39] |
| COM14 | Switzerland (–) | Biological reactor | [33] |
| COM15 | Belgium (Korbeek 3) | Maize field (soil surface) | [39] |

atrazine applications combined with other favourable factors [28]. Soils from Ireland had been receiving repeated and high loadings of simazine as a non-selective herbicide [13].

### *Obtaining the microbial communities*

A basal medium (BMA) containing only a liquid salts solution (NFB) and atrazine (20 mg $L^{-1}$) as sole source of N was used. The NFB solution was prepared according to Tomasek and Karns [36] and made of 995 ml of a buffered solution ($K_2HPO_4$ (4.34 g $L^{-1}$); $KH_2PO_4$ (3.4 g $L^{-1}$); $MgSO_4 \cdot 7H_2O$ (0.2 g $L^{-1}$); $CaSO_4 \cdot 2H_2O$ (0.062 g $L^{-1}$)) supplemented with 1 ml of a trace element solution [36]. When needed, the pH of this solution was adjusted to 6.9. The pesticide was first dissolved in 1 ml of methanol and then mixed with NFB. The basal medium obtained in this way was sterilised for 30 min at 121°C. HPLC analysis after autoclaving revealed that a minor part of the pesticide (less than 1%) was transformed to hydroxyatrazine (HA). Obtaining active microbial suspensions from soils started with an enrichment procedure. Soils were suspended in BMA (20 g of soil in 60 ml BMA). When the atrazine concentration was drastically reduced (10% or less of the initial concentration), the suspension was resuspended in fresh BMA. This process was repeated three times followed by a serial dilution procedure from which only the highest dilution exhibiting degradation of atrazine was kept. After this stage, microbial cultures were resuspended (1:10 v:v) every 2 weeks in fresh basal medium. This period of time was sufficient to allow atrazine degradation and development of all mixed cultures.

### *Atrazine biodegradation kinetics*

Kinetics of atrazine biodegradation was studied in 250-ml Erlenmeyer flasks containing 100 ml of BMA at 25°C, in the dark. Aliquots of 2 ml were regularly taken in order to carry out measurements of absorbance and HPLC analysis. Absorbance at 600 nm was measured using a Beckmann DU-8 spectrophotometer. HPLC analysis was carried out under isocratic conditions (methanol:water (5% acetonitrile) 60:40) using a 25-cm Alltima C18 5-$\mu$m column (Alltech Europe, Laarne, Belgium). These conditions allowed the detection of atrazine (ATR), hydroxyatrazine (HA), déethylatrazine (DEA) and déisopropylatrazine (DIA). The DT50 values were determined graphically from the degradation curves.

### *Biolog analysis*

Biolog GN microplates (Biolog, Hayward, CA, USA) containing 95 different carbon sources were inoculated with a suspension from each of the communities. These microbial populations were grown in BMA medium. After reaching stationary phase, they were diluted ten-fold prior to inoculation. Each community was tested in duplicate. Incubation conditions were 28 ± 1°C without agitation. Under these conditions, the $OD_{595\ nm}$ values of the inocula were between 0.02 and 0.04. Colour development in the wells was measured after 24, 48, 72 and 96 h using a microplate reader (Titertek Multiskan MCC/340, filter 595 nm).

Absorbance values reached a plateau after 72 h. Results obtained after this period of time were therefore used for data analysis. In order to compare the levels of absorbance registered for the different plates (as advised by Garland [14]), absorbance measurements were normalised using the formula:

$$W = (W_m - C)/\mathrm{MAX}(W_m - C)$$

where $W$ is the normalised absorbance, $W_m$ the measured absorbance of a given well, and $C$ the absorbance of the control well. The normalised absorbance for each well ($W$) is thus equal to the difference between its measured absorbance ($W_m$) and that of the control ($C$) divided by the greatest difference of absorbance developed by a well of the plate ($\mathrm{MAX}(W_m - C)$). The control well is inoculated with the microbial material but does not contain any carbon source. Consequently, $W$ falls in a continuous range from 0 (colour development identical to the control well) to 1 (maximum well colour development for the plate). Statistical analysis was carried out using the Statistica software package from StatSoft (Tulsa, OK, USA). Average $W$ values were used for multilinear regression analysis.

## Results and discussion

### *Atrazine biodegradation*

The three parameters measured while following the biodegradation of atrazine are summarized in Table 2. Figure 1 presents six communities representative of the different patterns registered. The disappearance of atrazine was in thirteen cases very rapid with almost total disappearance within the first 80 h. COM15 degraded atrazine the most rapidly (ATR-DT50 = 20 h) whereas COM10 was the slowest (ATR-DT50 = 163 h).

In two cases, the maximum concentration of HA reached a level higher than 15% of the initial atrazine concentration. In many cases, HA production and degradation were concomitant with atrazine dissipation. For COM2, COM11 and COM13, the concentration of HA increased only during the first phase of atrazine degradation. For these communities (and also COM3, COM4, COM5, COM6, COM8 and COM9, results not shown), the HA concentration remained rather low, not exceeding 10% of the initial ATR concentration. After this stage, HA generally dissipated within the same time as atrazine. But in three cases (COM5, COM11 and COM13), the disappearance of HA was not fully accomplished after dissipation of the parent compound, the metabolite being still present at levels around 10%. This residual HA was then only slowly degraded. COM15 has a totally different behaviour compared with the other communities. The HA reached a level of more than 65% after only 30 h of incubation; once the atrazine was extensively degraded, the accumulated HA was rapidly dissipated to reach a non-detectable level after 55 h. The key role of HA in the rapid atrazine biodegradation pathway [23,29] is thus confirmed. For none of the communities studied were DEA or DIA detected, whereas these two compounds have often been considered as the major metabolites of atrazine biodegradation [7,12].

The absorbance increase was always weak, which is in accordance with the low concentration of the organic sources present in solution. It can nevertheless reach 0.2 OD units. In all cases, most of the absorbance increase was concomitant with ATR and HA dissipation but in most cases a high level of atrazine could be dissipated without significant absorbance evolution (eg COM11 and COM13). Actually, only the steps following HA formation are likely to provide C and N for microbial growth. For some communities (COM2, COM15), a plateau of absorbance was observed when ATR and HA were dissipated indicating that the metabolic process was finished. For others such as COM6, absorbance continued to rise, indicating that some C and N sources were still present in the medium and that metabolic activity continued.

**Table 2** Atrazine DT50, $HA_{max}$, and ABS values determined for the 15 consortia

| Community | ATR DT50 (h) | $HA_{max}$ (% initial ATR) | ABS (600 nm, at $t = 196$ h) |
|---|---|---|---|
| COM1 | 46 | 14.0 | 0.20 |
| COM2 | 33 | 4.1 | 0.09 |
| COM3 | 42 | 7.0 | 0.10 |
| COM4 | 72 | 7.5 | 0.12 |
| COM5 | 45 | 23.0 | 0.20 |
| COM6 | 47 | 3.7 | 0.15 |
| COM7 | 59 | 8.9 | 0.16 |
| COM8 | 30 | 7.9 | 0.12 |
| COM9 | 27 | 9.5 | 0.19 |
| COM10 | 163 | 10.7 | 0.06 |
| COM11 | 56 | 13.4 | 0.22 |
| COM12 | 55 | 13.2 | 0.20 |
| COM13 | 66 | 9.1 | 0.08 |
| COM14 | 119 | 9.0 | 0.16 |
| COM15 | 20 | 67.2 | 0.09 |

## Biolog characterisation

### *Carbon source utilisation*

The following 24 substrates were found to be degraded by all 15 communities ($W>0.2$): Tween 40, Tween 80, l-arabinose, d-arabitol, cellobiose, d-fructose, d-galactose, $\alpha$-d-glucose, maltose, mannitol, sucrose, *cis*-aconitic acid, citric acid, d-gluconic acid, $\beta$-hydroxybutyric acid, $\alpha$-keto glutaric acid, d,l-lactic acid, quinic acid, l-asparagine, l-aspartic acid, l-glutamic acid, l-proline, l-pyroglutamic acid, and inosine.

Four substrates (acetic acid, $\alpha$-keto valeric acid, d-serine, and thymidine) were not degraded by any of the communities; 2,3-butanediol was degraded only by COM12; $\alpha$-hydroxybutyric acid by COM2. On the other hand, $\beta$-hydroxybutyric acid was degraded by every community and $\gamma$-hydroxybutyric acid by four communities (COM10, COM12, COM13, and COM14). Similar results were reported for these substrates [22] in studies on the characterisation of soil microbial communities.

The determination coefficients between the normalised absorbance obtained for the 95 Biolog wells inoculated with each of the different microbial communities were calculated. As a general observation, Biolog wells were weakly correlated to each other (90% of $r^2$ values below 0.4). Thus the set of C sources present on the GN Biolog microplates may be considered as satisfactory in view of the richness of information gathered and its power to discriminate between the different communities. Some substrates were highly correlated ($r^2 > 0.95$); they are characterised by similar chemical structures (glucose-1-phosphate and glucose-6-phosphate, d-alanine and l-alanine, $\alpha$-d-lactose and lactulose, d-galacturonic acid and d-glucuronic acid, l-asparagine and l-aspartic acid and l-glutamic acid).

### *Cluster analysis*

Figure 2 presents a tree diagram summarising the outcome of cluster analysis performed on the different communities. The first level of clustering is always between the duplicate microplates. The differences between duplicates are always smaller than between two plates inoculated with different communities. The Euclidean distance between two duplicates is generally around 0.5 and always lower than 1 whereas the distance between two communities is always higher than 1.1. Two clusters of bacterial communities are formed at a linkage distance below 1.3: cluster{COM1, COM4, COM5} and cluster{COM6, COM8, COM11, COM15}. These two clusters and COM12 are linked to each other at a distance smaller than 1.6. On the other hand,

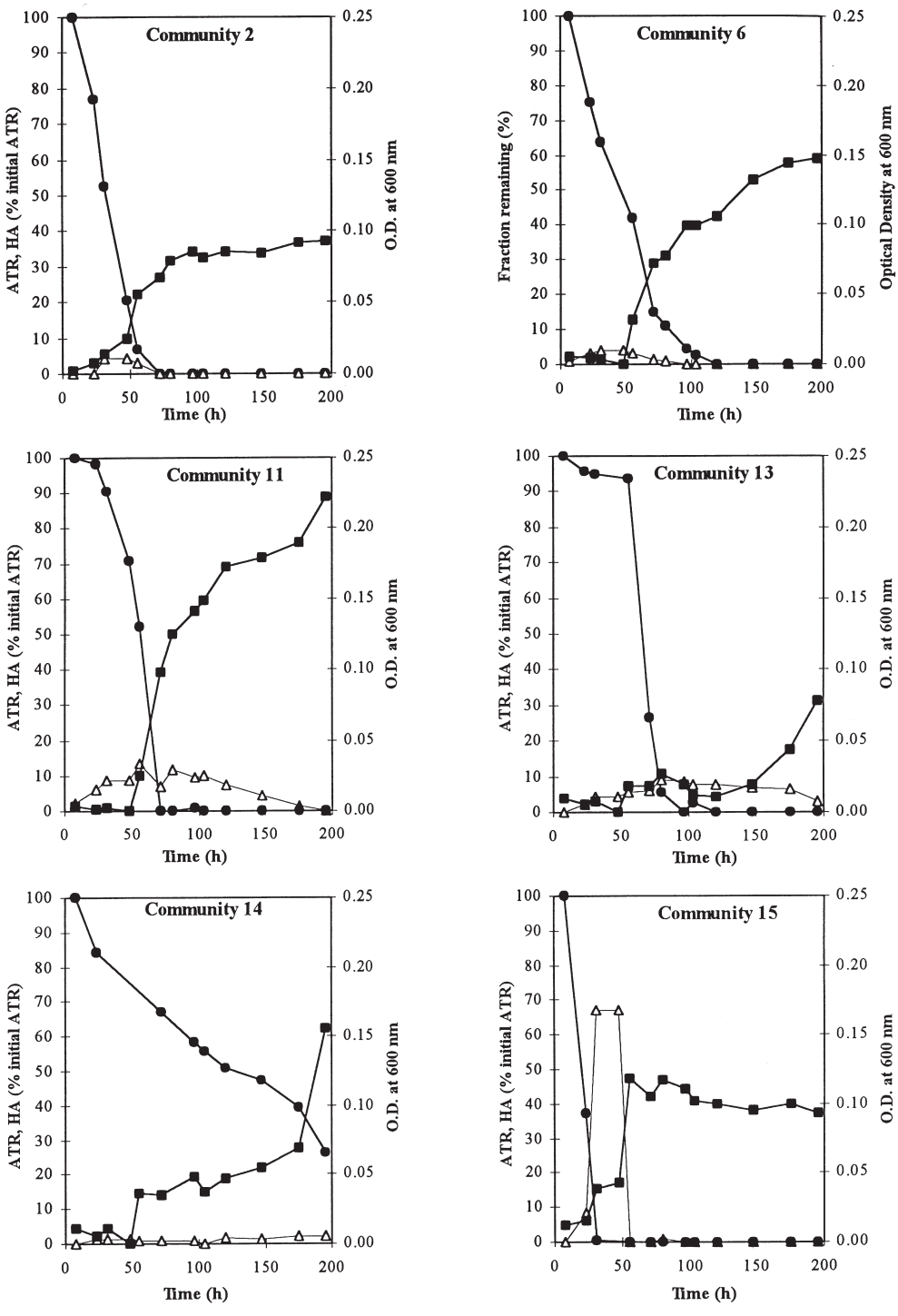


**Figure 1** Degradation of atrazine by six characteristic microbial communities. Atrazine (ATR, ●) and hydroxyatrazine (HA, Δ) are expressed as a percentage of the initial atrazine concentration. The optical density (■) was measured at 600 nm.

COM7, COM9, COM3, COM2, COM13, COM14 and COM10 exhibit a 'chain' type clustering. They differ strongly from each other as well as from the rest of the communities since their Euclidean distances from any other community are higher than 1.6.

The three communities obtained from subsurface sediments (COM11, COM12, and COM13) are not clustered. On the contrary, their spreading throughout the different clusters indicates that their metabolic abilities are as diverse as those observed amongst the communities originating from the soil surface. COM13 was able to degrade only a fairly restricted number of C sources, whereas COM11 exhibited the second widest variety of substrate catabolism. COM11 and COM12 degraded many of the substrates that were recalcitrant to the majority of the other communities, COM12 being the only consortium able to degrade 2,3-

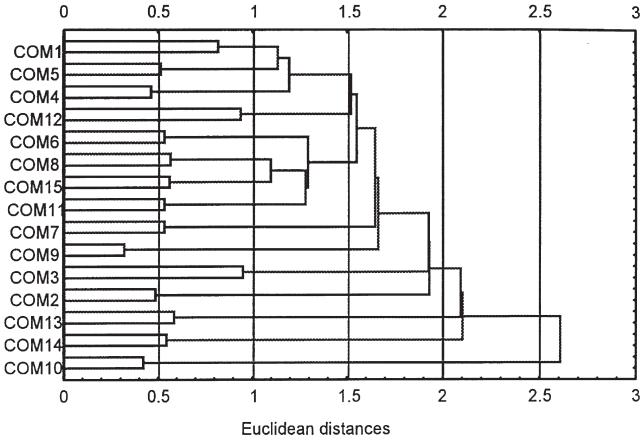


**Figure 2** Tree diagram of the metabolic fingerprints of the 15 bacterial communities using the Single Linkage method.

butanediol. This community also exhibited some metabolic activity with propionic acid and d,l-$\alpha$-glycerol phosphate that were amongst the most recalcitrant C sources. Noteworthy also is the fact that the two communities originating from Irish soils (COM6 and COM7) were not clustered. The first homogeneous cluster formed is {COM1, COM4, COM5} wherein all the three communities were obtained from Belgian maize fields (topsoils). Within the second cluster, soils from very different origins are grouped together (COM6, COM8, COM11, COM15).

## Link between atrazine biodegradation and Biolog characterisation

None of the substrates present on the Biolog microplates possesses a chemical structure close to atrazine. Nevertheless, the four aromatic compounds (inosine, thymidine, uridine and urocanic acid) exhibit an heterocyclic structure containing nitrogen. Among those, inosine was degraded by all communities and shows some structural similarities with hydroxyatrazine. However, atrazine-degrading communities did not exhibit specific abilities to develop on all four heterocyclic compounds, thymidine being recalcitrant to all communities and uridine being degraded by only half of the communities studied. At this stage, no link between the ability to degrade these compounds and atrazine can be established.

The correlation between atrazine DT50 and any $W$ value was low ($r < 0.8$). In order to study whether a stronger link could be established between the $W$ values of some Biolog wells and the atrazine DT50 values, a forward stepwise multilinear regression approach was used. Considering the 15 consortia, no satisfactory relation ($F > 20$) could be established (data not shown). This could be attributed to the heterogeneity of the DT50 values and especially to two outlying communities (COM10 and COM14) exhibiting much higher values, 163 and 119 h respectively. In order to obtain more significant relationships between atrazine-degradation abilities and Biolog substrate use by the various communities, it was decided to limit the data set to the soil communities able to degrade atrazine at a high rate (DT50 $<$ 100 h); hence, COM10 and COM14 were discarded. The peculiar characteristics of the two excluded communities (COM10 and COM14) were also apparent in the cluster analysis (Figure 2) since they are the last two communities to cluster to the others. This restricted data set thus showed DT50 ranging from 20.3 to 70.6 h (Table 2).

A satisfactory description of the DT50 values (considered as the dependent variable) was obtained after the incorporation (as independent variables) of $W$ values corresponding to four different substrates:

$$\mathrm{DT50} = -72.4 - 75.4\ W_{\text{Turanose}} + 380.7\ W_{\text{L-glutamic acid}} - 235.8\ W_{\text{Citric acid}} - 40.3\ W_{\text{L-pyroglutamic acid}}$$

$$n = 13;\ R = 0.97;\ F(4,8) = 46.7$$

where $n$ is the number of observations, $R$ the coefficient of multiple regression, and $F$ = Regression Mean Square/Residual Mean Square. This descriptive equation is significant at a high probability level ($P < 0.00001$), the contribution of any substrate being significant at $\alpha < 0.05$. Using the same data set, no significant description could be obtained when the maximum level of HA ($HA_{max}$) or of absorbance ($ABS_{max}$) was taken as an independent variable.

The four substrates used in the multilinear regression (turanose, l-glutamic acid, citric acid, and l-pyroglutamic acid) do not exhibit chemical similarities with atrazine. No direct link with the atrazine catabolic pathway can thus be established. The link observed between atrazine DT50 and Biolog measurements could be explained in another way: the genes coding for atrazine degradation can be found amongst different species of different genera [6,11,29] and the Biolog substrates selected by the stepwise multilinear regression could thus reflect the type of competent strains present within each community. The DT50 of atrazine could thus be related to activities of some selected members characterised by a specific substrate utilisation.

## Conclusion

Atrazine biodegradation can be carried out by a wide diversity of microbial consortia. Although hydroxyatrazine was the sole metabolite detected, the different patterns of atrazine dissipation, hydroxyatrazine accumulation and dissipation as well as biomass production reveal that the communities studied possess different biodegradation abilities. Functional diversity of these consortia could not be linked with their origins and communities obtained from subsurface soils may exhibit as much functional diversity as those originating from soil surface samples. A multilinear relation describing the atrazine DT50 values could be drawn from the Biolog measurements. It is suggested that this relationship could reflect the type of competent microorganisms present within each community.

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