RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2007) 10:209-215
DOI: 10.2436/20.1501.01.29 ISSN: 1139-6709 **www.im.microbios.org**

INTERNATIONAL MICROBIOLOGY

Analysis of *s*-triazine-degrading microbial communities in soils using most-probable-number enumeration and tetrazolium-salt detection

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Received 24 June 2007 · Accepted 31 July 2007

Summary. A simple and sensitive method for the detection and enumeration of microbial *s*-triazine-degrading microorganisms in soil was designed. The procedure is based on the ability of some microbes to use *s*-triazines, such as simazine, atrazine, and cyanuric acid, as sole nitrogen source. It employs the respiration indicator 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) to detect metabolic activity and the most-probable-number (MPN) enumeration in microtiter plates. The method was used to identify simazine- and cyanuric acid-degrading activities in agricultural soils treated with the herbicide simazine. The MPN-TTC method showed that the number of simazine- and cyanuric acid-degrading microorganisms increased four weeks after the herbicide simazine had been applied. [**Int Microbiol** 2007; 10(3):209-215]

Key words: *Pseudomonas* sp. ADP · *s*-triazine · simazine · atrazine · tetrazolium salt · herbicides · biodegradation

Introduction

s-Triazines, including simazine (2-chloro-4,6-bis[ethylamino]-*s*-triazine) and atrazine (2-chloro-4-ethylamine-6-isopropylamino-*s*-triazine), have been used worldwide in weed control for over 40 years [11,17]. These synthetic chemicals are the most widely applied herbicides in the USA, where 38,000 tons of atrazine and simazine were purchased during 2001 [Environmental Protection Agency (2004) Pesticides industry sales and usages: 2000 and 2001 market estimates. EPA, Washington, DC, USA]. In Chile, the growth of agro-industry is reflected by the 350 tons of atrazine and simazine that were sold in 2004 [Servicio Agrícola y Ganadero (2006) Declaración de ventas de plaguicidas año 2004. Ministerio de Agricultura, Santiago, Chile, pp 1-152]. Despite their widespread application, these herbicides are toxic and present environmental as well as health-related risks [8,21,24], necessitating the remediation of herbicide-polluted sites. Various strategies for restoring contaminated soils employ microbial degradation [6,28]. Whereas microorganisms involved in biodegradation—mainly bacteria, but also fungi [25]—generally use the pollutant as carbon source, simazine and atrazine might serve as nitrogen sources for bacteria [12,26,27,30].

To establish bioremediation processes that remove these compounds, the detection of active indigenous microbial

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communities able to degrade *s*-triazines is required. Typical procedures for soil analysis combine chemical and molecular approaches [3,9]. Methods that monitor chemical parameters, such as high-pressure liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and gas chromatography with electron capture detection (GC-ECD), have been used to identify and quantify *s*-triazine herbicides in soils [13,18,31]. Molecular biology approaches assess the presence and diversity of microorganisms and the catabolic genes for *s*-triazine degradation [27]. However, none of these methods is able to determine microbial catabolic activities. A simple laboratory method for the detection and enumeration of *s*-triazine-degrading bacteria is thus desirable to develop strategies for the bioremediation of polluted soils.

This work describes a simple and sensitive microbiological method for most-probable-number (MPN) enumeration of *s*-triazine-degrading microorganisms in soil. The method is based on the ability to detect reduction of the tetrazolium salt 2,3,5-triphenyl-2H-tetrazolium chloride (TTC), as a colored formazan, by the metabolic activity of microorganisms that use *s*-triazines such as simazine, atrazine, and cyanuric acid as sole nitrogen source. MPN enumeration, which is used to estimate microbial population size based on a process-related attribute [15], measures only live and active organisms. The number of viable cells can be determined by measuring turbidity or colorimetric changes under selective enrichment conditions. The MPN-TTC method tested in this study was able to enumerate *s*-triazine-degrading cells based on the reduction of TTC by microbial dehydrogenase activities. The technique was applied to measure the activities of simazine- and cyanuric acid-degrading microorganisms in agricultural soils treated with *s*-triazine herbicides.

Materials and methods

Bacterial strains and growth conditions. *Pseudomonas* sp. strain ADP [19] was used as the *s*-triazine-degrading microorganism for MPN-TTC assays and was grown at 30ºC. *Escherichia coli* strain DH5α (Invitrogen, USA) grown on Luria Bertani media at 37ºC was used as a negative control. The minimal medium for selective growth contained (per liter) 0.5 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, and 10 ml of trace elements containing (per liter of stock solution): 1 g $MnSO_4 \cdot H_2O$, 0.9 g $CaSO_4$, 0.8 g $FeSO_4 \cdot 7H_2O$, 0.2 g $CoSO_4 \cdot 7H_2O$, 0.2 g $ZnSO_4 \cdot 7\ H_2O$, 0.03 g $CuSO_4 \cdot 7H_2O$, 0.02 g Na_2WO_4, 0.02 g Na_2SeO_3 and 0.02 g $NiCl_2 \cdot 6\ H_2O$. Citrate (15 mM) and succinate (28 mM) were used as the carbon sources. Simazine and atrazine (≥99% pure, Labor. Dr. Ehrensdorfer-Schäfers, Germany), at a final concentration of 2 mM, and cyanuric acid (Merck, Germany), at a concentration 1 mM, were used as the nitrogen sources [7].

Sampling site and soils treatment. A commercial avocado plantation in which simazine had been applied for almost 20 years was selected for soil sampling. Soil samples were extracted (in triplicate) using sterile steel cylinders inserted in a soil auger. For each sample, microbial *s*-triazine catabolic activities and simazine concentration were determined. Ten grams of horizon top core soil (0–10 cm) from each sample were diluted in 90 ml of sodium phosphate buffer (1.2 mM, pH 7.0). The samples were homogenized by treating the initial dilution with an automatic stomacher (Stomacher 400 circulator) for 5 min. Five ten-fold serial dilutions were prepared, each containing inocula from 1×10^{-4} g to 1×10^{-7} g of soil.

TTC reduction and formazan production. Validation of the method for the detection of *s*-triazine-degrading cells based on the reduction of TTC (Merck, Germany) was established with the strain *Pseudomonas* sp. ADP. The bacterium was inoculated in three minimal media (20 ml) cultures containing simazine, atrazine, or cyanuric acid as the nitrogen source and incubated at 30ºC. At different times, samples (0.5 ml) were incubated with TTC (0.01%) in the dark for 4 h and then diluted with ethanol (1 vol) to dissolve insoluble formazan. The amount of TTC formazan produced was determined by measuring absorbance at 490 nm (A_{490}) with a lambda 25 Perkin-Elmer spectrophotometer. *s*-Triazines were quantified in the samples as described below. Cultures without inocula were used to measure background TTC formazan production.

MPN-TTC method for the detection of *s*-triazine-degrading cells. Validation of the method for the enumeration of *s*-triazine-degrading cells using MPN-TTC was established in microtiter plates with the strain *Pseudomonas* sp. ADP. Samples with different inocula were prepared by suspending a colony of *Pseudomonas* sp. ADP in 9 ml of minimal medium (without *s*-triazine). Ten-fold serial dilutions to extinction were prepared (in triplicate) to obtain the minimal inoculum. MPN detection of *Pseudomonas* sp. ADP was carried out by inoculating 100 µl from a dilution in each of five wells of a microtiter plate containing simazine, atrazine (previously dried in a sterile flow-hood), or cyanuric acid. The microtiter plates with the serially diluted inocula were incubated at 30ºC for one week. At the end of the incubation period, TTC was added (0.01% final concentration) to each well. A blank, without inoculum, was used as the control. After 4 h of incubation at 30ºC, positive wells were determined by visualization of the color change and by measuring TTC formazan formation. Samples were dissolved with ethanol (1 vol) and TTC formazan produced was measured as the A_{490} with a Lambda 25 Perkin-Elmer spectrophotometer. The number of positive wells per serial dilution was recorded and estimations of viable *s*-triazine-degrading cells of *Pseudomonas* sp. ADP were obtained using the MPN calculator-program [16]. For *E. coli* DH5α (negative control), the same procedure was used. The sensitivity of the method was determined by calculating the inoculum size for each sample at time 0 based on the number of colony-forming units (CFU), with minimal media agar plates containing simazine as the sole nitrogen source. The plates were incubated at 30ºC for one week, after which the CFU were counted.

Procedures for the detection of simazine- and cyanuric acid-degrading cells in soils using MPN-TTC. Five wells of a microtiter plate containing the culture media and simazine or cyanuric acid were inoculated with each dilution of soil samples. The microtiter plates were incubated for one week at 30ºC. Sterile distilled water was added to wells to compensate water lost by evaporation. At the end of the incubation period, TTC (0.01% final concentration) was added and the positive wells were recorded as previously described. The number of cells/g of soil was calculated using an MPN calculator-program [16].

Analysis of *s*-triazines. Simazine and atrazine concentrations were determined by a spectrophotometric method [5,7]. Samples from microtiter plate wells were centrifuged (10,000 ×*g*) at 4ºC. The simazine and atrazine concentrations in supernatants were quantified by measuring absorbance at 225 nm (A_{225}) [5,7]. The concentration of cyanuric acid in aliquots (20 µl) from supernatants was determined by HPLC. A 5C18-MS-II column and a mobile phase of 5 mM KH_2PO_4/K_2HPO_4 buffer solution (pH 7.0) at a flow rate of 1.0 ml/min were used [5,7]. Simazine, atrazine, and cyanuric acid were quantified by comparison with calibration curves of authentic stan-

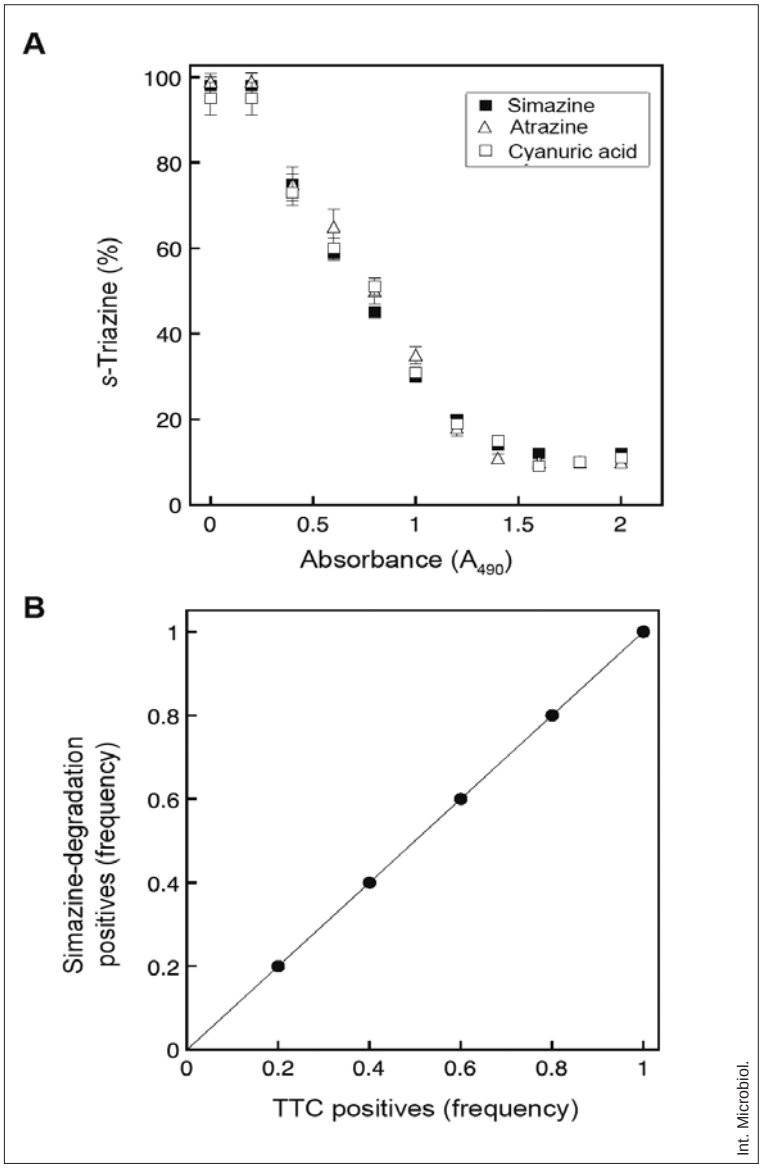

Fig. 1. Detection of *s*-triazine-degradation and TTC reduction by *s*-triazine-degrading cells of *Pseudomonas* sp. ADP. (**A**) Comparison of *s*-triazine degradation and formazan production according to TTC reduction by *Pseudomonas* sp. ADP. TTC formazan was determined by measuring the A_{490}. (**B**) Correlation between the frequencies of microtiter wells positive for simazine degradation and those positive for TTC reduction. Wells with formazan production $A_{490} \geq 0.4$ were scored as positive for TTC reduction [25]. Wells with simazine degradation ≥25% were scored as positive. Data represent five independent experiments.

dards. Controls without inoculum were used to determine the *s*-triazine concentration at the end of incubation.

The simazine concentration in soil samples was determined by extraction with methanol (80%), separation with solid-phase extraction on C18 columns (Accubond, Agilent, 0.5 g) and quantification by GC-ECD. To standardize the extraction methods and quantification, simazine of high purity (99.6% pure, Accustandard, USA) was used.

Results and Discussion

Detection of *s*-triazine-degrading cells. Figure 1A shows that TTC formazan production (A_{490}) by *s*-triazine-degrading cells of *Pseudomonas* sp. ADP correlated with the degradation of *s*-triazines. Figure 1B shows the correlation between the frequencies of microtiter wells positive for TTC reduction and those positive for simazine degradation. Wells that were positive for reduced TTC correlated with those that were positive for simazine degradation.

In MPN enumeration, selective growth conditions and serial dilutions are used to estimate the number of viable cells, even when present at low concentrations, and thus microbial population size. With this approach, together with the MPN-TTC method, in which TTC is reduced to a colored formazan, the specific presence of viable *s*-triazine-degrading cells was determined. The culture medium used for the enrichment of *s*-triazine-degrading cells contains, in addition to citrate as carbon source, an excess (28 mM) of the electron donor succinate, which activates microbial dehydrogenases [15]. TTC successfully competes with NAD^+ and $NADP^+$ for

Table 1. Counts of *s*-triazine-degrading cells as determined by the MPN-TTC method**,** % remaining *s*-triazines, and TTC formazan absorbance

		Simazine			Atrazine			Cyanuric acid		
Strain	log CFU	MPN[a]	%[b]	A_{490}[c]	MPN	%	A_{490}	MPN	%	A_{490}
Pseudomonas sp. ADP	2.5	2.3 (±0.2)	12	1.8	2.5 (±0.2)	10	2	2.4 (±0.2)	12	1.7
	1.5	1.2 (±0.2)	12	1.7	1.3 (±0.2)	11	1.8	1.2 (±0.2)	10	1.7
	0.5	0.4 (±0.2)	12	1.8	0.5 (±0.2)	10	1.8	0.4 (±0.2)	11	1.8

[a]MPN enumeration of s-triazine-degraders (average of triplicate samples ±1 standard deviation).
[b]Percentage of *s*-triazine remaining in the culture medium at the end of the incubation period (average of triplicates).
[c]Change in TTC reduction measured by TTC formazan absorbance (A_{490}) at the end of the incubation period (average of triplicates).

electrons shuttling between ubiquinone and cytochrome *b* in the electron-transport chain. As TTC is an electron acceptor, it is reduced to a red formazan that can be detected colorimetrically [29]. Tetrazolium salts are toxic for microorganisms at the concentrations used in viability assays [14]; therefore, in this study, TTC salts were added at the end of incubation to exclude toxicity and alterations in microbial community structure.

Results concerning the detection of *s*-triazine-degrading cells, using *Pseudomonas* sp. ADP and the MPN-TTC method, are shown in Table 1. MPN was estimated considering the dilution factor and the inoculum in each well. The growth from one *Pseudomonas* sp. ADP cell per well was determined to be sufficient to produce a positive colorimetric response.

Fig. 2. Combination of TTC positives in a microtiter plate at different soil dilutions by the TTC-MPN method. A soil sample from an agricultural field treated with simazine was analized. The positive colorimetric reaction indicates formazan production due to TTC reduction.

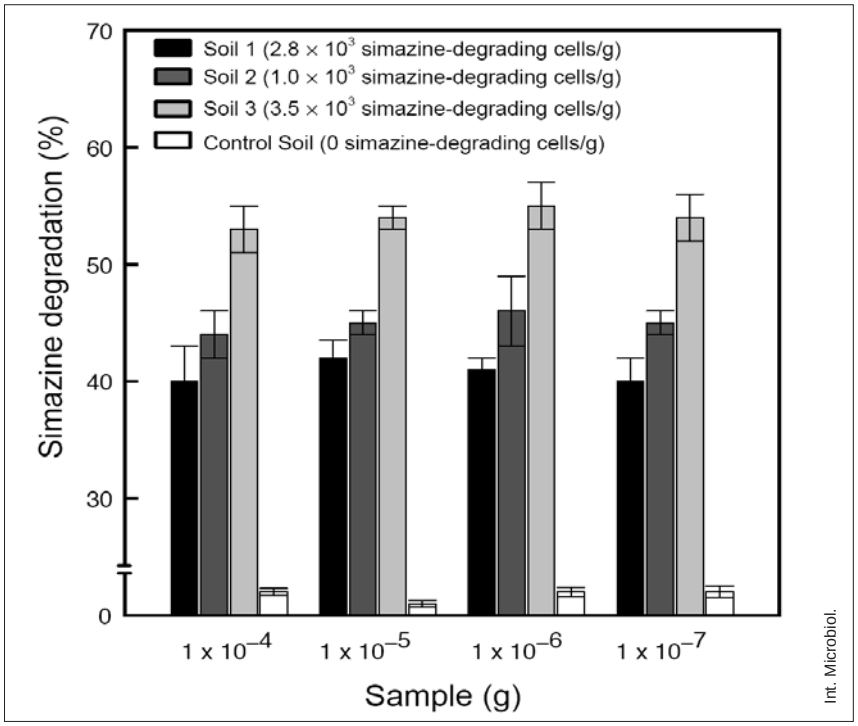

Fig. 3. Simazine degradation by microorganisms in soils enumerated with the MPN-TTC method. Soils 1, 2 and 3 were obtained from agricultural fields annualy treated with simazine. A sample from a non-agricultural field was used as control. The values represent the mean of three independent experiments ±1 standard deviation.

In order to determine whether a positive colorimetric response was representative of active *s*-triazine-degrading cells, simazine, atrazine, and cyanuric acid were measured at the end of the incubation period. Data shown in Table 1 validate that TTC formazan production reflected *s*-triazine degradation by active *s*-triazine-degrading cells. Similar concentrations of remaining *s*-triazine were obtained at the end of the incubations regardless of the size of the inoculum. MPN enumeration with serial dilution assays is designed to detect bacterial growth produced even by one viable cell. Therefore, the *s*-triazine degradations observed at the end of incubation in dilutions with low cellular densities were independent of inocula size and were related to the ability of *Pseudomonas* sp. ADP to growth and degrade *s*-triazines. Results from the negative control *E. coli* DH5α showed that there were no false-positive reactions under the experimental conditions tested; in those cultures, no *s*-triazine degradation was observed. This supports the conclusion that the method is appropriate for the recovery, detection, and enumeration of *s*-triazine-degrading microorganisms.

Detection of simazine-degrading activities in soils using MPN-TTC method. Figure 2 illustrates a representative MPN-TTC soil assay. Positive wells were visualized by a colorimetric change in a well containing culture medium with simazine as the sole nitrogen source. TTC-positive wells were confirmed by measuring formazan production at A_{490}. As seen in the figure, the number of TTC positives decreased with increasing soil dilutions. The presence of viable simazine-degrading cells was revealed by the reduction of TTC to a colored formazan due to microbial dehydrogenases activities. These respiratory activities are present in aerobic, facultative and anaerobic microorganisms [14]. The number of simazine-degrading cells was estimated by MPN considering three dilutions with corresponding decreases in the number of TTC-positive wells. Each well was analyzed for TTC reduction and simazine was quantified at the end of the incubation period. For each soil sample, wells containing inocula of four different concentrations were analyzed for simazine degradation. Figure 3 shows the simazine degradation by simazine-degrading cells from the four different soil samples. Consistent with the statistical basis of MPN (probability and confidence intervals) [4], no significant differences in the numbers of simazine-degrading-cells were detected in soils 1, 2, and 3. Since simazine-degrading activities in dilutions (to extinction) were inocula-independent, it can be concluded that the differences in simazine degradation observed in the soil samples were related to the metabolic abilities of indigenous simazine-degrading communities. Bacteria (mainly) and fungi [25] contribute to the degradation of *s*-triazines in soils. Since the MPN-TTC method employs a selective growth system, it probably underestimates the simazine-degradation potential of microorganisms in soil [25].

Simazine- and cyanuric acid-degrading activities in soil exposed to simazine. The MPN-TTC method was used to assess the simazine- and cyanuric acid-degrading activities in soil in response to the herbicide simazine. The agricultural field selected had been treated for many years with simazine. The soil samples analyzed were extracted in the field, from the first horizon top soil (0–10 cm), after simazine application. The simazine concentrations were calculated in each soil sample by extraction with methanol, separation on C18 columns, and quantification by GC-ECD. In freshly applied herbicide soil, 1.1×10^2 and 6.0×10^3 simazine- and cyanuric acid-degrading cells, respectively, were detected per gram of soil. At the time of this initial assay, the concentration of simazine in soil was 6.6 ppm. Four weeks after simazine application, 2.8×10^3 simazine-degrading cells and 5.6×10^4 cyanuric-acid-degrading cells per gram of soil were detected by the MPN-TTC method. The simazine concentration in soil at this second time point was 0.9 ppm. The half-life of *s*-triazine in soil ranges from 8 to 99 days, depending on soil management and physicochemical variables [26].

MPN enumeration estimates the size of microbial populations based on the recovery of cells with specific and selective abilities. In this study, the MPN-TTC method took advantage of the selective ability of microorganisms to degrade an *s*-triazine and to use it as sole nitrogen source. When employed in combination with an adapted metabolic marker, such as TTC, the procedure is simple and sensitive. These metabolic markers record the activity of the electron-transport chain. Since this activity is related to microbial degradation [10], the method is specific. Tetrazolium salt allows the detection of microorganisms that use polycyclic aromatic hydrocarbons as carbon source [15], quantification of microbial activity in petroleum [20] and at sites contaminated with heavy metals [23], as well as evaluation of the antimicrobial activities of natural products [2,22] and the toxicity of polluted soils [1]. The present work shows that MPN enumeration, combined with the TTC tetrazolium salts as indicator, is a useful, sensitive, specific, and cost-effective method for the detection of microbial populations able to use *s*-triazines as nitrogen source.

Acknowledgements. The authors are grateful for the financial support of grants EU ICA4-CT2002-10011, USM 130522, Milenio P04/007-F, DIPUV 18-05 and FONDECYT 3050052. M.A.D. acknowledges a postdoctoral position from grants EU ICA4-CT2002-10011 and FONDECYT 3050052 (Fondo Nacional de Investigación Científica y Tecnológica, Chile). The authors thank Edward Moore for helpful suggestions and critical reading of the manuscript and Larry Wackett for providing *Pseudomonas* sp. strain ADP.

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