Monitoring atrazine uptake by a new fluorometric assay

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We report here a new fluorometric method for monitoring atrazine uptake using a strain of *Acinetobacter radioresistens*. Around 40% decrease in the relative fluorescent intensity of the culture supernatant and a corresponding increase in the fluorescence in cell biomass was observed after 18 h of growth on the fluorescing substrate, which was indicative of the rapid entry of the compound into the bacterial cell. This fast and safe method is a new application in the field of environmental biotechnology and can be used as an additional approach to monitor uptake of other toxic compounds by different microorganisms and to screen for different pollutant degraders.

Keywords. *Acinetobacter radioresistens*, atrazine uptake, fluorescein 5-isothiocyanate, fluorometric assay.

MICROORGANISMS with their large metabolic repertoire play a major role in the breakdown and mineralization of the various pollutants, including the recalcitrant pesticides being introduced into the fragile environment. However, not only their degradation but also the rapid detection of organisms capable of acting on such pollutants is of vital importance if rapid bioremediation strategy has to be employed for pollutant removal and environment clean-up, especially in cases of massive accidental spills. The first step, however, includes monitoring of the uptake of the pesticide under study by the degrading organism. This study introduces a simple method for the detection of pesticide uptake by degrading microorganism(s). This method is based on the measurement of change in fluorescence of the target compound and has been successfully described here using atrazine (2-chloro, 4-ethylamino, 6-isopropylamino-s-triazine) tagged with FITC (fluorescein 5-isothiocyanate) as the test compound.

Atrazine is a triazine group of pesticides used since the last 30 years for selective weed control in crops such as sorghum, maize, sugarcane, citrus, etc.[1]. It is an endocrine-disrupting chemical reported to be carcinogenic and causing prostrate, ovarian, breast and uterine tumours and also leukaemia and lymphoma (www.nrdc.org/health/pesticide/natrazine-asp). Various organisms capable of degrading atrazine have been isolated. Predominant among them is *Pseudomonas* sp., on which maximum work has been reported followed by *Rhodococcus* sp.[2–4]. In the studies mentioned above and several others, methods such as radioactive carbon (^{14}C) assay, HPLC and GC have been employed[5–7]. Radioactive procedures are complicated and require caution while handling the radioactive compounds, whereas HPLC and GC require precise and quantitative pesticide-extraction procedures. In addition, these methods are time-consuming.

The method proposed here is based on the change in the relative fluorescence of culture supernatant with time by the uptake of atrazine–FITC conjugate during bacterial growth. Fluorescence-based studies have long since been a part of protein and DNA analyses. In this study, however, a method is being proposed for the detection of uptake of an environmental pollutant.

For the construction of FITC–atrazine conjugate, first a mercaptopropionic acid derivative (MPAD) of atrazine was synthesized using a method reported elsewhere[8,9]. This derivative was then conjugated to FITC through a linker, ethylenediamine. The reaction to form FITC–atrazine from its MPAD derivative has been described in Figure 1.

Specificity of the altered atrazine molecule was confirmed by spectrophotometric comparison of growth (OD 600 nm) of A6 on atrazine and MPAD separately. A6 was grown in minimal medium (as described earlier) with 50 mg/l of the individual compounds as carbon and energy source. Similar growth comparison was done between atrazine–FITC, atrazine and FITC separately. However, due to substrate constraint, in this experiment, 2 mg/l of atrazine–FITC and FITC was used. Atrazine also was used here at the same concentration (2 ppm) to maintain uniformity. *Escherichia coli* V517, a non-atrazine degrader, was taken as a negative control.

Assay for atrazine uptake included monitoring fluorescence change in culture supernatant and cell biomass upon growth of the organism on fluorescent atrazine as the sole source of carbon and energy in minimal medium, without any other carbon or energy source. An *Acinetobacter radioresistens* strain isolate from the authors' laboratory, capable of growth on atrazine was used in the study[10]. In one set, the organism was grown in minimal medium containing FITC-labelled atrazine as carbon source, without any organism acting as control. In a separate set, A6 was grown in minimal medium with only FITC as the sole carbon and energy source. Minimal medium containing only FITC (5 μg/ml) and no organism was used as control. Working solution of atrazine–FITC was prepared upon dilution from its stock and correction for dilution in the media was done by monitoring relative fluorescence intensity, such that the concentration of the two substrates was comparable. Samples were drawn at 6 h interval, cells removed by centrifugation and fluorescence reading of the supernatant was taken with controls of the respective sets used for calibration using a spectrofluorimeter (Kontron SFM 25, Switzerland). In all the cases, excitation was done at 492 nm, with emission readings

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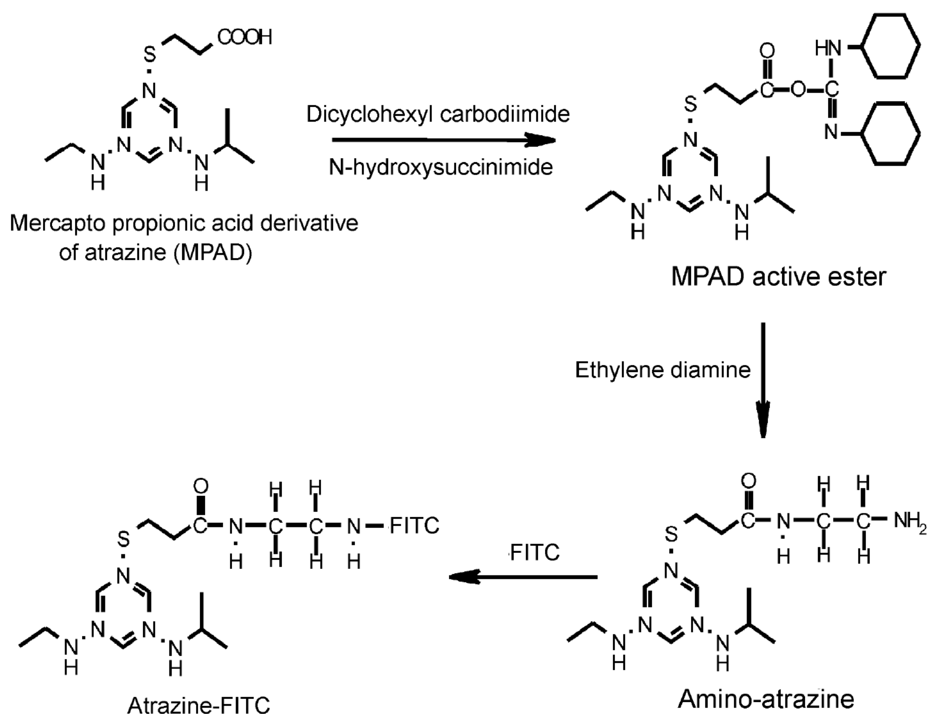

Figure 1. Reaction mechanism for the synthesis of atrazine–FITC conjugate from its MPAD derivative.

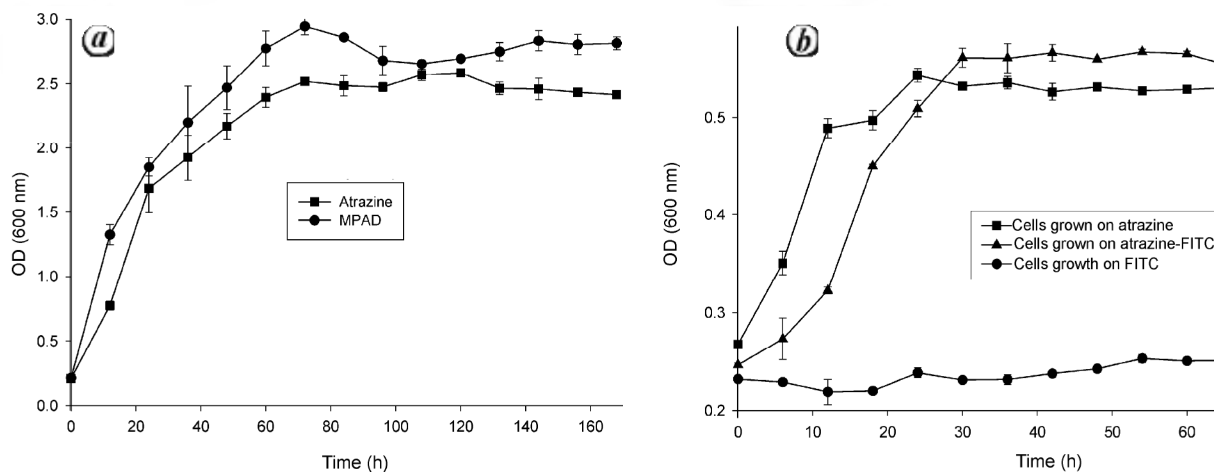

Figure 2. Growth comparison of strain A6 on (*a*) atrazine and mercaptopropionic acid derivative of atrazine, and (*b*) FITC-tagged atrazine and FITC.

taken at 518 nm. The experiment was performed in triplicate under controlled temperature conditions (25 ± 0.5°C) and mean of the sets taken for analyses.

Further confirmation of atrazine uptake by this method was done taking the fluorescent reading of the extract of the cell biomass obtained after growth in the atrazine–FITC conjugate. The growth medium and amount of substrate were kept constant as before. After different time intervals, cells were harvested by centrifugation and washed thoroughly five times (0.01 M sodium phosphate buffer (pH 7.0) and 0.85% NaCl solution), before finally suspending them in minimal medium suitable for sonication. Sonication was performed by a 30 s pulse with 30 s break in between, for a total time of 40 min. Cell debris was removed and fluorescence of the cell lysate was taken at the same parameters as mentioned above.

Confocal microscope (LSM 510, Zeiss, Germany) was used for visual confirmation of the uptake of the fluorescing substrate. Cells obtained after growth on FITC-tagged atrazine were immobilized on polylysine-coated coverslips,

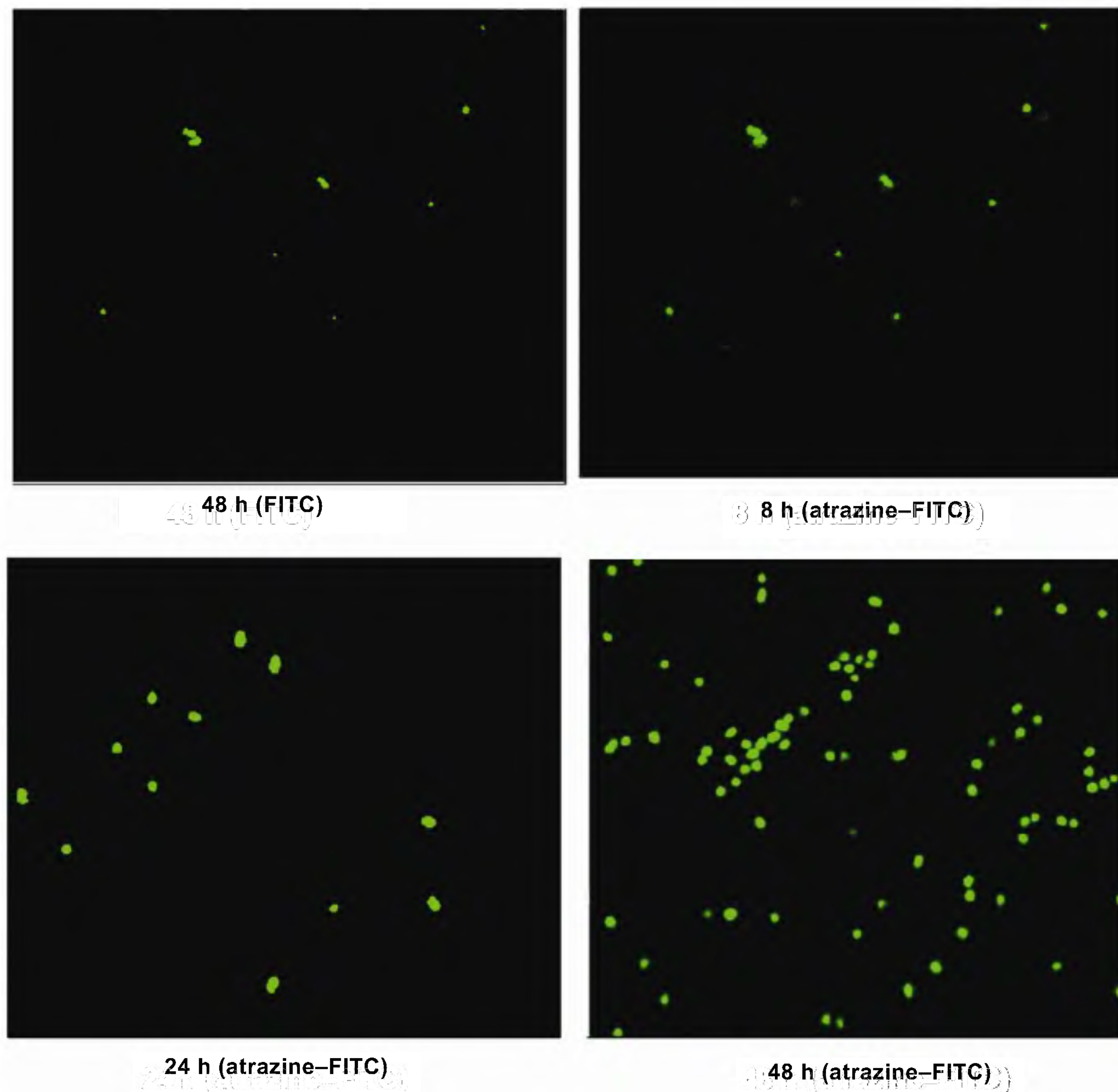

Figure 3. Confocal images of strain A6 recovered after different growth times.

mounted over slides and examined under the microscope under oil immersion. Cells recovered at 0, 8, 12, 24 and 48 h of growth were used for viewing. Cells recovered after 48 h exposure in only FITC containing minimal medium were also processed similarly and acted as negative controls.

The first step in the construction of the fluorescent test compound required the construction of a fluorescent atrazine molecule using FITC. Tagging of FITC to atrazine produced a fluorescent molecule with no major change in its properties, as proved by monitoring the growth of the organism on different forms of altered substrate, viz. MPAD and FITC–atrazine and comparing it with the growth profile on atrazine alone (Figure 2 *a* and *b*). A marginal difference in the growth profiles was observed when the strain A6 was grown on FITC-atrazine and on atrazine alone (Figure 2 *b*). The strain failed to grow solely on FITC, while almost comparable growth was obtained on the other substrates tested. *E. coli* V517, taken as negative control failed to grow in any of the substrates. Antibody specificity against tagged molecule was the final confirmation. The hapten (MPAD) has been used in conjugation with a carrier protein to raise highly specific antibodies against atrazine[11]. The same hapten–

FITC conjugate has been used to develop a highly specific fluoro-immunoassay for atrazine in our laboratory (unpublished data). It was therefore clear that alteration of the substrate molecule produced no major change in its biochemical properties.

Atrazine-contaminated soil isolate, *A. radioresistens* was found to be capable of growth on FITC-tagged atrazine when used as the sole carbon and energy source in minimal medium. A sharp decrease in the relative fluorescence of atrazine–FITC conjugate to approximately 40% of control, was observed around 18 h of growth that coincided with the point of entry of cells in the log phase of their growth. Minimal medium alone did not contribute to fluorescence and its minimal quantum yield alone at the excitation and emission wavelengths was corrected for, while taking readings of the samples. The decrease in fluorescence became stable after this initial sharp dip, signifying probably the saturation of the cells with the amount of tolerable atrazine molecules. There was no change in the fluorescence of the atrazine–FITC conjugate in control medium without any cells. Similarly, no change in fluorescence was observed when cells were grown on FITC alone, showing thereby that it is atrazine–FITC uptake and not methanol or FITC utilization/adhesion that causes a decrease in fluorescence. This result also rules out the possibility of A6 using FITC alone as the carbon and energy source. Similarly, *E. coli* V517 (MTCC 131), a non-atrazine degrader, also failed to show any growth. A corresponding increase in fluorescence was observed in the assay of fluorescence of cell lysate of A6, confirming the uptake of FITC-labelled atrazine inside the cells.

When viewed under confocal microscope, compared to control, cells recovered after 24 h of growth in the atrazine–FITC conjugate showed intense fluorescence, with cells recovered after 48 h of growth showing maximum fluorescence (Figure 3). Although there was no growth of A6 on FITC alone, cells recovered after 48 h were viewed but no fluorescence in the marginal cells present was observed, ruling out the possibility of the adhesion of fluorescent compounds to bacterial cells.

The results obtained here establish the fact that uptake of atrazine can be monitored easily by monitoring the decrease in fluorescence of FITC tagged to it. Although the altered molecule was vastly different from the parent compound in terms of size, no change was observed in the biochemical properties of the organism towards any of the altered substrates. For the degradation of any compound, its fast and efficient uptake is the first important step. Hence quick monitoring of uptake assumes importance if pollution by a compound is more, and fast screening of the concerned organisms is the key factor. This study opens up a new area of pesticide degradation analysis, whereby the uptake of any pesticide by its degrading microorganism can be monitored using this simple and easy fluorescence based method. This technique is much safer than other existing methods and can be used as an additional method for fast screening of pesticide uptake in the test medium.

In conclusion, we have developed a novel, fluorescence-based technique for monitoring atrazine uptake by degrading microorganisms, which has the potential to be applied for studying the uptake of other pesticides and toxic pollutants by different microorganisms.

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