JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2007,** *55,* 3823−3828 **3823**

Quantitative Determination of Diuron in Ground and Surface Water by Time-Resolved Fluoroimmunoassay: Seasonal Variations of Diuron, Carbofuran, and Paraquat in an Agricultural Area

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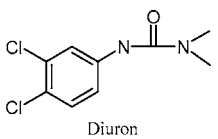
The aim of this research is to develop an ultrasensitive time-resolved fluorescence immunoassay (TR-FIA) for herbicide diuron in water samples. This method appears to be a promising approach, instead of conventional analytical techniques, in the screening procedure of organic pollutants because it is simple, rapid, and specific, and it does not require sample preconcentration or cleanup. Lanthanide chelate used as label allows to achieve sensitivity even 10 times higher than most of the other techniques. It has been applied to monitoring diuron contamination in specimens collected along a year in an agricultural area. The water specimens were collected monthly from lake, well, and irrigation ditch in the agricultural area south of Milan. Assay was performed using diuron-specific polyclonal antibody raised in sheep; as fluorescent marker, we used rabbit antisheep IgG conjugated with a chelating molecule complexed with Eu^{3+}. The compound 4-(3-(3,4-dichloro-phenyl)-1-methyl-ureido)-butyric acid (CPD) was synthesized and conjugated with bovine serum albumin (BSA) to prepare a solid phase. Sensitivity achieved was 20 ng L^{-1} below the European Community limits. Paraquat (PQ) and carbofuran (CF) presence in the same samples has been also evaluated in a similar way, using immunoassays with time-resolved revelation systems. Diuron concentration shows a peak coinciding with a peak of carbofuran during summer periods. The peak of diuron was 65 pg/mL in June and 180 pg/mL in September in ditch and lake water samples, respectively; carbofuran concentration was higher than diuron in all samples: a carbofuran peak was revealed in September and October resulting in 87 ng/mL. Herbicide paraquat was not detectable in any assayed sample.

KEYWORDS: Diuron; urea herbicides; immunoassay; europium chelate; time-resolved fluorescence; pesticides

INTRODUCTION

The herbicide diuron is a substituted urea compound used to control a wide variety of annual and perennial weeds or as antifouling biocides (*1*, *2*). This compound inhibits the photosynthesis by blocking electron transport, through preventing the CO_2 fixation and limiting the adenosine triphosphate production. It is very soluble in water and very stable in soil (*3*): in soil, in which the processes of degradation are biotic transformations, it exhibits 200 days half-life, while in water (in which hydrolysis or photolysis is major route of degradation) it has 90 days half-life (*4*). The reduction of dissolved oxygen concentration induced by diuron led to decomposition of photosynthetic microorganisms and modification of microbial flora of aquatic environment. Diuron is highly toxic to aquatic invertebrates (*5*); by preventing algal cellular reproduction, diuron indirectly limited the release of dissolved organic substances by phytoplankton (*6*, *7*). Furthermore, even if low lethality is observed in adult fishes, dose-dependent delay is present in hatch (*8*). In addition, genotoxic effects on plant chromosomes such as inhibition of mitotic index and induction of chromosome aberrations have also been reported (*9*).



Diuron
3-(4-Dichloro-phenyl)-1,1-dimethylurea

The herbicide PQ (1,1′-dimethyl-4,4′-bipyridinium) is a quaternary nitrogen compound, active as redox drug inhibiting the reduction of NADP to NADPH during photosynthesis. It is used as a nonselective contact herbicide. It is known to generate superoxide anions in mitochondria and cytosol of yeast and

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10.1021/jf063442o CCC: $37.00
Published on Web 04/13/2007

mammalian cells leading to the formation of several reactive oxygen species (*10*, *11*).

The pesticide CF (2,3-dihydro-2,2-dimethyl-7-benzofuranyl-methylcarbamate) is worldwide used to control soil and leaf-feeding insects and nematodes. CF toxicity is very high in human and birds, as it is a potent inhibitor of cholinesterase by binding to the serine residues (*12*). It is widely used and can be found as pollutant in the air, water, soil, and foods.

Determination of pesticide residues in water from field application is generally restricted to the area around the site of application. However, contamination may be more extensive and may reach aquifer-confining systems, which requires large-scale monitoring programs.

The hazard is most commonly associated with long-term exposure to these contaminants. In particular, as for all pesticides, it is necessary to evaluate the exposure to diuron in the general population, especially in occupationally exposed people and residents in contaminated sites.

Therefore, several analytical approaches such as liquid chromatography, gas chromatography, mass spectrometry (*13*−*19*), electrophoresis (*20*, *21*), and immunoassay (*22*−*24*) have been described for detection of this compound. Conventional methods consist of separative techniques to isolate the target compound from other more concentrated compounds. Interferent peaks in the chromatograms and noisy baseline must be removed to allow sensitivity, which is lower than immunological methods.

Therefore, in conventional approach, the samples must be singly processed, which is laborious and time-consuming. As the number of controls required increases, the screening of many samples becomes very expensive. Furthermore, each step of an analytical process introduces errors into the final result. Immunological methods appear to be a promising approach for the development of a screening procedure. These methods cover a wide concentration range and do not require preconcentration or cleanup. They are simple, rapid, sensitive, and specific. Furthermore, many samples can be analyzed simultaneously in a short incubation time.

For instance, in the assay described in this paper, a single operator can analyze about 100 water samples simultaneously within 2 h including the reading time. If lanthanide chelates are used as labels, the immunoassay results are even 10 times more sensitive than most of the other techniques. Lanthanide chelates show narrow and strong emission bands around 600 nm and an exceptionally long decay time (up to a millisecond instead of nanoseconds of most molecules), which allow the elimination of the high background of the fluorescent labels.

In this paper, we reported a time-resolved fluoroimmunoassay (TR-FIA) to determine diuron concentrations in ground and surface water.

Thus, this study has a twofold objective. The first goal is to develop a method in which we combine advantages of immunological methods and sensitivity of time-resolved fluorescent revelation system; the second objective is to monitor seasonal variations of diuron concentrations in ground and surface water in an agricultural area south of Milan.

MATERIALS AND METHODS

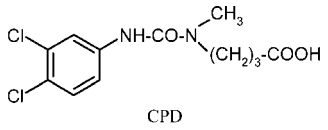
Materials. All of the chemicals, including standard diuron and the diuron-related cross-reacting compounds (linuron; chlortoluron: isoproturon; 3,4-dichloroaniline; metobromuron; chlorosulfuron: metolachlor), bovine serum albumin (BSA), and rabbit antisheep IgG were obtained from Sigma-Aldrich (Milan, Italy); diuron-specific polyclonal antibody raised in sheep (obtained using diuron conjugated to thyroglobulin as an immunogen) was purchased from Guildhay Ltd (Guildford, U.K.).

Apparatus. A single-photon-counting time-resolved fluorometer (1232 DELFIA Fluorometer; Wallac, Turku, Finland) was used to measure fluorescence.

Specimen Collection. The surface water specimens were collected from Fagnana lake (Buccinasco, Milan, Italy), from an irrigation ditch, and from a well in the agricultural area located south of Milan. Sampling was made monthly from September 2005 to July 2006 at the same places and under the same conditions. The water samples were collected in glass vials, were filtered, and were stored at 4 °C until use.

Syntheses. *1. BCPDA and IgG-(BCPDA)$_n$.* Europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) was synthesized from 2,9-dimethyl-4,7-diphenyl-[1,10]phenanthroline in a three-step procedure as described in detail by Evangelista et al. (*26*). Rabbit antisheep IgG-(BCPDA)$_n$ conjugate was prepared using 2 mg of affinity-purified IgG dissolved in 2 mL of 0.1 mol L^{-1} sodium carbonate buffer (pH 9). A solution of 500 μg of BCPDA in 100 μL of ethanol was then added, and the mixture was incubated for 30 min at room temperature. The labeled IgG was separated on Sephadex G-50 column, eluting with 0.05 mol L^{-1} Tris-HCl buffer (pH 7.3). The value for n ($\cong$36) was assessed as the absorbance of BCPDA at 325 nm; $\epsilon = 1.52 \times 10^4$ M^{-1} cm^{-1}.

2. CPD Hapten. 4-(3-(3,4-Dichloro-phenyl)-1-methyl-ureido)-butyric acid (CPD) was obtained according to the method described for isoproturon (*27*) with the following modifications. A 4-methylamino-butyric acid hydrochloride solution was prepared by adding 8.5 mmol of this compound to 10 mL of water containing 18.5 mmol sodium hydroxide; 8.4 mmol of 3,4-dichlorophenylisocyanate was added to the solution under fume hood and was stirred for 2 h at room temperature at which time the solution pH was 11. The solution was filtered, and the filtrate was acidified to pH 3 by adding 2 mol L^{-1} HCl. The resulting precipitate was filtered, was washed with H_2O till pH 5, and then was dried over $CaCl_2$ to yield 890 mg of product. This compound was chromatographically unequivocal on silica gel F_{254} TLC plates; $R_f = 0.8$ (methanol:chloroform 1:4); mp 149−151 °C.



CPD

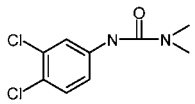
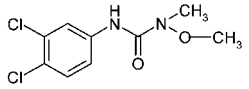
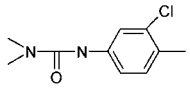
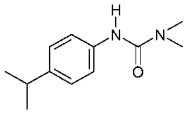
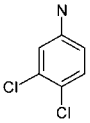
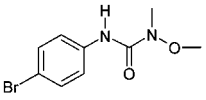
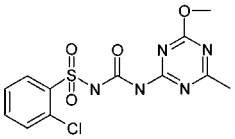
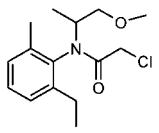
4-[(3-(3,4-Dichloro-phenyl)-1-methyl-ureido)-butyric acid

3. BSA−CPD. Fifteen micromoles of CPD was dissolved in 400 μL anhydrous dimethylformamide and was cooled to 4 °C before the addition of 5 μL tri-*n*-butylamine. After 10 min, 2 μL isobutylchloroformate was added, and the solution was left for 20 min at 4 °C before being added to 8 mg BSA dissolved in 2.5 mL 50% dimethylformamide in aqueous 0.9% NaCl. The pH was adjusted to 8.5 using 1 N NaOH, and the reaction mixture was left overnight at 4 °C before being eluted on Sephadex G-50 with 0.05 M NH_4HCO_3 at room temperature, lyophilized, and stored at 4 °C.

Preparation of Solid Phase. Polystyrene microtiter wells were coated overnight at 27 °C with 200 μL of 0.1 M carbonate buffer, pH 9.0, containing 10 μg/mL of BSA−CPD conjugate. The microtiter wells were washed with carbonate buffer, and a second coat was made with 250 μL of 2% BSA solution in the same buffer for 4 h at 27 °C. The wells were washed five times with 0.05 M Tris-HCl buffer pH 7.5 containing 0.9% NaCl and 0.05% NaN_3 and were stored dry at 4 °C until use.

Antibody Titer Evaluation. The antibody was assayed by incubation in wells coated with the BSA−CPD compound: 100 μL of serial dilutions of antiserum in buffer 0.05 M Tris-HCl buffer pH 7.5 containing 0.9% NaCl (1:250, 1:2500, and 1:25 000) was applied to the coated wells, and the antibody bound to the solid phase was detected by means of rabbit antisheep immunoglobulin G conjugated to BCPDA. After washing, 150 μL of dissociation solution containing 4 mol L^{-1} urea, 1% sodium dodecylsulfate, and 10^{-6} mol L^{-1} Eu^{3+} was added to

Table 1. Percentage of Cross-Reactivity of Diuron-Related Compounds with Diuron-Specific Antibody Calculated at 50% of Fluorescence Signal Reduction

Compounds	% Cross-Reactions
 Diuron *3-(4-Dichloro-phenyl)-1,1-dimethylurea*	100
 Linuron *N'-(3,4-Diclorophenyl)-N-methoxy--N-methylurea*	88
 Chlortoluron *3-(3-Chloro-4-methyl-phenyl)-1,1-dimethyl-urea*	10
 Isoproturon *3-(4-Isopropyl-phenyl)-1,1-dimethylurea*	5
 3,4-Dichloroaniline *3,4 dichloro-phenylamine*	0.1
 Metobromuron *N'-(4-Bromophenyl)-N-methoxy-N-methylurea*	< 0.01
 Chlorosulfuron *1-(2-Chlorophenylsulfonyl)-3-(4-methoxy--6-methyl-1,3,5-triazin-2-yl)-urea*	< 0.01
 Metolachlor *2-Chloro-N-(2-ethyl-6-methyl-phenyl)--N-(2-methoxy-1-methyl-ethyl)-acetamide*	< 0.01

each well. Fluorescence was measured after 10 min at the excitation wavelength of 345 nm. The delay time was 400 μs after excitation, the emitted light being read at 615 nm. The dilution giving 50% of

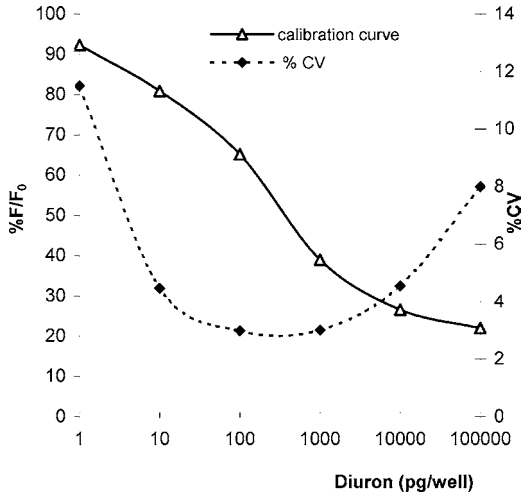


Figure 1. Dose–response curve and precision profile (percent coefficient of variation) for TR-FIA of diuron. F and F_0 are expressed as cps. Each point represents the mean of eight determinations in duplicate.

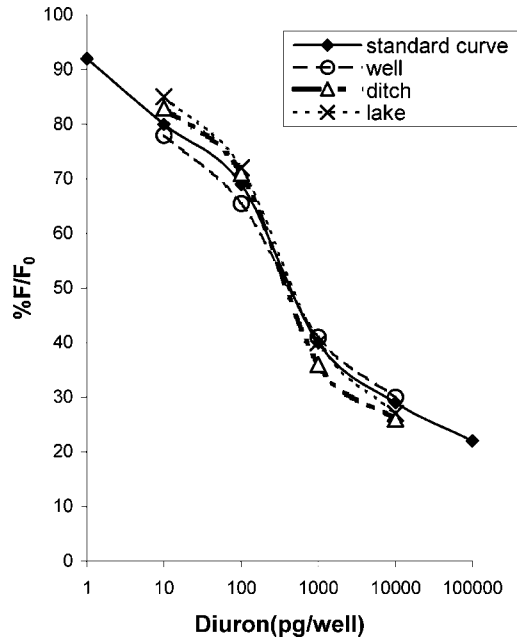


Figure 2. Parallelism of the assay expressed as logit–log function for well, ditch, and lake samples spiked with different amount of diuron. F and F_0 are expressed as cps (means of three determinations run in duplicate).

fluorescence intensity in comparison with 100% of saturated solid-phase fluorescence was chosen as working titer.

TR-FIA. TR-FIA was performed with the diuron standard dissolved in tap water. A hundred microliters of serial dilutions of standard solution between 1 pg and 100 ng was transferred in duplicate into polystyrene microwells coated with BSA−CPD conjugate. Specific antibody was diluted at a working titer of 1:7500 in buffer 0.05 M Tris-HCl solution, pH 7.5, containing 0.9% NaCl and 0.2% BSA; 50 μL of antibody solution was added to all of the wells other than that used for the blank evaluation, to which the same volume of buffer was added. Assay was performed directly in the water by applying 100 μL of samples in duplicate instead of the standards. Matrix effect was assayed by recovery evaluation of 2, 10, 100, and 1000 pg of standard diuron added to 100 μL of well, ditch, and lake water (winter month samples) with undetectable diuron concentration. The wells were washed, and 150 μL of buffer containing an excess of rabbit antisheep IgG labeled with BCPDA was added and incubated for 30 min. After

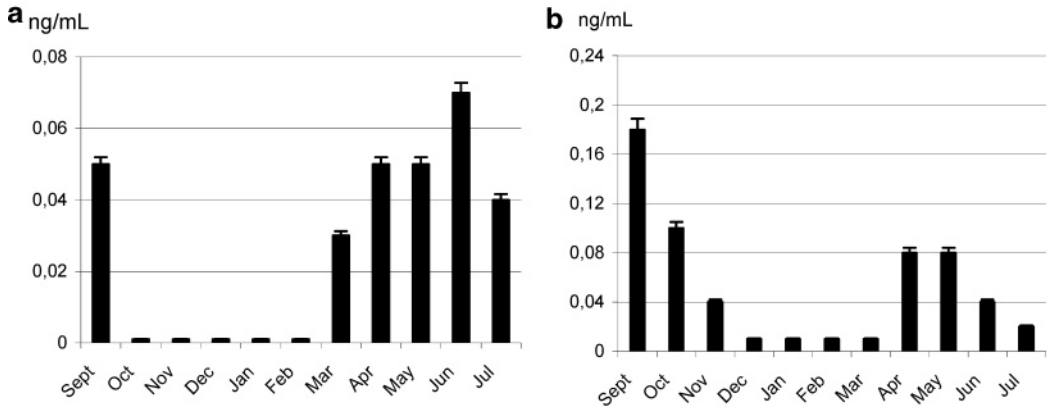


Figure 3. (**a**) Concentrations of diuron (ng/mL) in the ditch water samples in different months of 2006 (bars correspond to the standard deviations). (**3b**) Concentrations of diuron (ng/mL) in the lake water samples in different months of 2006 (bars correspond to the standard deviations).

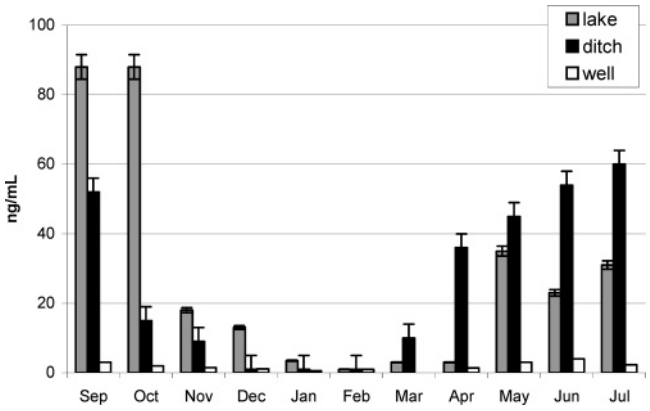


Figure 4. Concentrations of carbofuran (ng/mL) in water samples of lake, ditch, and well in different months in 2006 (bars correspond to the standard deviations).

washing, 150 μL of dissociation solution was added to each well and fluorescence was measured as described in Antibody Titer Evaluation section.

Paraquat (PQ) and Carbofuran (CF) Analyses. The method described for diuron determination shows characteristics like those described for PQ. PQ analysis was performed in the same water samples using TR-FIA with the same europium chelate as described in detail in the literature (*25*).

CF determination was carried out by immunoassay with Tb^{3+}/dipicolinic acid complex as time-resolved revelation systems, according to the previously published method (*28*).

RESULTS AND DISCUSSION

Antibody Specificity. Specificity of the polyclonal antibody was evaluated by assaying the cross-reactions of diuron-related compounds, calculated at 50% of fluorescent signal reduction against the 100% of standard diuron. As reported in **Table 1**, linuron shows a high cross-reaction value; the other structurally related compounds did not interfere in the assay.

Calibration Graph and Validation. The antibody dilution giving 50% of fluorescence intensity in comparison with 100% of saturated solid-phase fluorescence was chosen as the working titer. Dose−response curve is shown in **Figure 1**. The graph was obtained by averaging eight individual curves normalized by reporting fluorescence values as % F/F_0 where F was the mean of counts/s (cps) for each standard and F_0 was the mean of cps for zero diuron concentration; blank value (3100 $\pm$ 278 cps) was subtracted.

Assay sensitivity was good as shown by a high slope of calibration curve. The detection limit of the method was determined by calculating the minimum amount of diuron that could be significantly distinguished from zero (mean binding at zero dose at 3 times the standard deviation (SD)). This value, calculated from three curves prepared in duplicate, was 2 pg/well. Evaluation of recovery in different water matrixes was carried out in well, ditch, and lake samples taken in winter (January) with undetectable diuron amounts. Samples were spiked with 10, 100, 1000, and 10 000 pg of diuron: the three curves resulted parallel to the standard curve obtained in tap water (**Figure 2**).

Seasonal Variations in Water Samples. Because of its widespread use in agriculture, its solubility in water diuron should be assayed in agricultural countries to verify seasonal variations during to the application periods. We used the described assay to evaluate diuron concentration monthly in 2006, between September and July, in agricultural country near Milan. The presence of pesticide carbofuran and herbicide paraquat was also evaluated. Analytical results are summarized in graphs reported below. Concentrations of diuron are reported in **Figure 3a** and **Figure 3b**, for ditch and lake samples. In well samples, concentration was below detection limits.

Seasonal profile of carbofuran concentration in Fagnana lake, ditch, and well is shown in **Figure 4**. Variations in different summer months should be explained by taking into account the rainfall data of the studied period. The occurrence of rainfall facilitates the transport of pesticides from the site of application to the nearest water courses and afterward to the lake. Both the diuron and carbofuran concentrations in ditch raise a peak in June and July, while in lake concentrations they increase in the following months (September and October), after the rainfall.

Herbicide paraquat was not detectable in any assayed sample, which suggests that it was not applied in the considered area. On the other hand, also diuron concentration is very low. As it is present in very high dilution in aqueous samples, diuron is a good model for the application of the described method. In these conditions, the assay by common instrumental methods is impractical, because all conventional techniques are less sensitive than required. These techniques need complex sample treatments. The successful use of extraction and cleanup methods depends on many parameters, such as the choice of solid phase and polarity of the solvents, and requires expert technicians. Therefore, internal standard controls are necessary to monitor the recovery. Furthermore, individual procedure must be used for each sample. Immunoassays are a valid alternative. However, even if immunoassays are rather widely used in clinical

chemistry, they have not yet gained a similar diffusion in environmental and food analysis. The detectability of labels is one of the most critical factors which limits sensitivity of immunoassay. The development of fluorescent labels provides an important simplification of immunological procedures. Their usefulness has, however, been limited by the high-background fluorescence always present in the measurements, which seriously limits the sensitivity of the assay. Chemiluminescent signals also present similar problems. Some methods such as flow-injection immunosensor assay are very interesting because they facilitate automation, but their sensitivity results are reduced for the reasons reported above.

This problem can be overcome by the use of time-resolved fluorescence. This technique takes advantage of the emission characteristics of europium and terbium chelates (narrow and strong emission bands around 600 nm and an exceptionally long decay time), which allow the elimination of the high background of the fluorescent labels. The method reported in this paper uses this kind of label. In this way, many advantages were achieved. The BCPDA chelate is fluorescent in aqueous solution, so it is possible to determine fluorescence directly in aqueous samples. This allows the achieving of a good sensitivity at very high dilution and is suitable for a rapid, simple, and cheap screening. Furthermore, the reagents used can be easily obtained. The high degree of sensitivity makes this assay useful for checking diuron levels also in drinking water. Since high-performance liquid chromatography (HPLC) was unable to detect the analyte in the assayed concentrations, TR-FIA was validated by determining diuron recovery at different concentrations and matrix effects in different samples. The obtained values showed a good assay performance. The specificity always proved satisfactory with the exception of linuron cross-reactivity. In the presence of linuron, TR-FIA will thus overstimate the diuron. For samples showing high values of diuron concentrations, it should be important to individuate each specific species of herbicide. In this case, the sample will be further processed with a conventional method. This disadvantage could be overcome by the development of more specific hapten derivatives to use in antibody production.

Although diuron and carbofuran were detected in many samples during the summer periods, their concentrations were always below the available toxicity levels. So, in this case, no sample needs further control by HPLC or other instrumental methods.

This study suggests that the use of these pesticides under conditions employed does not result in concentrations harmful to the aquatic environment.

ACKNOWLEDGMENT

The authors are grateful to Dr. G. Morano, Municipal Education Department, Buccinasco (Milan, Italy), and to Dr. M. Marchese, Technical Institute G. Feltrinelli, Milan, Italy, for supplying the sample.

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Received for review November 28, 2006. Revised manuscript received March 14, 2007. Accepted March 14, 2007.

JF063442O