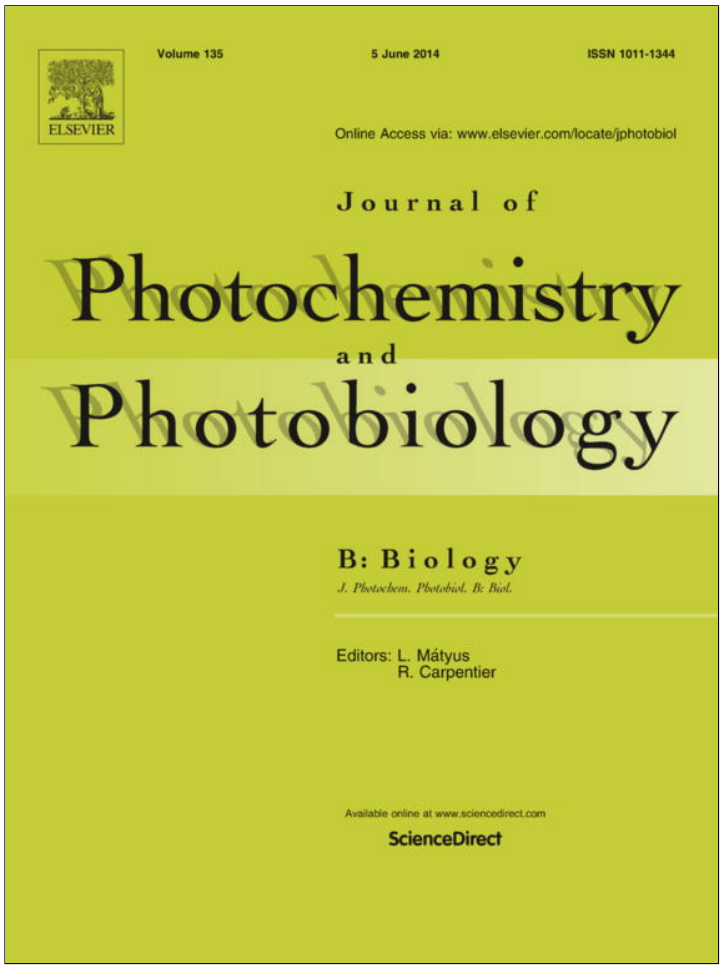

Journal of Photochemistry and Photobiology B: Biology 135 (2014) 48–54

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

On the natural fate of maleic hydrazide. Kinetic aspects of the photochemical and microbiological degradation of the herbicide

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ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form 5 March 2014
Accepted 7 April 2014
Available online 18 April 2014

Keywords:
Biodegradation
Humic acid
Maleic hydrazide
Photodegradation
Riboflavin

ABSTRACT

Kinetic and mechanistic aspects of the photochemical and microbiological degradation of the herbicide Maleic Hydrazide (MH) have been studied.

Riboflavin (Rf, vitamin B_2) was employed as a main photosensitizer whereas Humic Acid (HA) was included as a second sensitizer in order to more closely simulate natural environmental conditions. MH quenches excited singlet and triplet states of Rf, with rate constants close to the diffusion limit. The herbicide and dissolved molecular oxygen competitively quench triplet excited Rf. As a consequence the reactive oxygen species (ROS), superoxide radical anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and singlet molecular oxygen ($O_2(^1\Delta_g)$) are produced by electron- and energy-transfer processes, respectively, as demonstrated by auxiliary experiments employing selective auxiliary quenchers and the exclusive $O_2(^1\Delta_g)$ generator Rose Bengal (RB). As a global result, the photodegradation of Rf is retarded, whereas MH is degraded by the generated ROS. The bacteria *Pseudomonas aeruginosa* (Ps) and *Bacillus subtilis* (Bs), recognized as contaminants surface-water and soil and microbial antagonists of phytopathogenic, were used in the microbiological experiments. Results of the individual incubation of both bacteria in in the presence of MH indicate a stimulation on the Ps growth, implying the biodegradation of the herbicide, whereas MH only exerted a bacteriostatic effect on Bs.

1. Introduction

Many agricultural biocides of profuse use have molecular structures with a six-membered nitrogen-containing aromatic heterocycles. Significant amounts of these compounds could contaminate surface waters and soils [1,2,3]. MH (Scheme 1), a plant growth regulator and herbicide that acts by inhibiting cell division in plants, belongs to this class of chemicals [4]. High doses of its potassium salt appear to be genotoxic. Besides, some relatively recent laboratory studies demonstrated that the pesticide may cause liver and kidney toxicity in mice [5]. In this context, all possible information about the fate of the pesticide under environmental conditions is very important, due to its profuse employment in crop protection and other practical applications.

After release into the environment, pesticides in general may have different destines, being unavoidable its immediate exposition to natural light and native microorganisms. As a consequence, systematic studies on the kinetics of photo- and bio-degradation pathways of such a contaminants are topics of growing interest that could help to foresee the environmental decay of these compounds [1, 6, 7].

According to our knowledge, only two works were published on direct MH photoirradiation. Stoessl, in 1964, described the photodegradation of MH in aqueous solution, being a number of acidic compounds, including succinic, maleic and fumaric acids, identified as photoproducts [8]. Recently, Reva et al., studied the UV-induced photoisomerization of maleic hydrazide. UV irradiation of matrix-isolated maleic hydrazide induced two isomerization processes yielding the dihydroxy tautomer and N-aminomaleimide [9].

The optical absorption spectrum of MH is extended up to 370 nm. This fact predicts a limited extent for the direct degradation of the herbicide by the action of direct sunlight irradiation. For this class of compounds an interesting possibility of photodeg-

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http://dx.doi.org/10.1016/j.jphotobiol.2014.04.002
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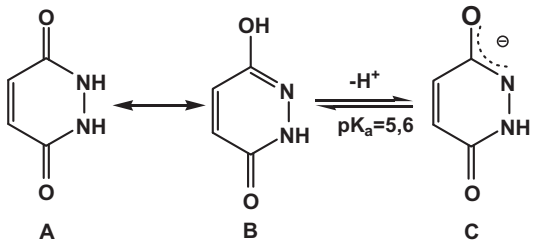

Scheme 1. Accepted tautomeric forms and acid–base equilibria for MH.

radation to be explored is through a photosensitized process. This means the irradiation of the polluted medium in the presence of a photosensitizer, using light of wavelengths longer than those absorbed by the pollutants. This procedure takes advantage of the presence in surface natural waters of some colored compounds that can act as the primary environmental light-absorbers. Traces of sensitizers are usually present in water courses, lakes and seas. Among them, HA and, in special, the pigment Rf are relevant in the sensitized photooxidation of contaminants [10,11,12]. Both HA and Rf generate $O_2(^1\Delta_g)$, with quantum yields of 0.04 (upper limit) and 0.47, respectively [13,14]. The vitamin also generates $O_2^{\cdot-}$ with a quantum yield of 0.009 [15].

As an attempt to standardize the photodegradability of MH upon Rf-photosensitization, its stability was compared with those of two recognized molecules: phenol (ph), a paradigmatic water-contaminant model compound and tryptophan (trp), one of the most studied biological targets in photodynamic action.

Regarding the possible bio-degradation route of MH, many soil and water microorganisms have the ability to degrade certain pesticides [16]. Since the disappearance of contaminants due to microbial degradation obeys to different general conditions, the feasibility and effectiveness of such a process must be studied as a particular case for the different contaminant-microorganism combinations.

On this basis, the purpose of the present research work was to evaluate the rates, and mechanism involved in the photo and bio-degradation of the pesticide MH under simulated environmental conditions.

In doing this, we employed Rf and HA as potential natural sensitizers for the degradation of MH. For the biological assays the microorganisms Ps and Bs were used. Ps is a natural contaminant bacteria of surface waters whereas Bs is frequently found in soils and marine waters [17,18,19]. Both bacteria can exert antagonistic effects on certain phytopathogens, through the production of bacteriocins or antibiotics that adversely affect the development thereof, being able to be employed in the biological control of plagues [20–22].

2. Materials and methods

2.1. Materials

Maleic hydrazide, Riboflavin, superoxide dismutase (SOD) from bovine erythrocytes and catalase (CAT) from bovine liver, were purchased from Sigma Chem. Co., Rose Bengal, furfuryl alcohol (FFA), deuterium oxide (D_2O; 99.9 atom% D), phenol, L-tryptophan and humic acid were from Aldrich (Milwaukee, WI, USA). Sodium azide (NaN_3) was from Merck. The chemicals K_2HPO_4, Na_2HPO_4, KH_2PO_4, NH_4Cl, NaCl, NaOH, $MgSO_4$, $CaCl_2{\cdot}6H_2O$, $NaHCO_3$, and $FeCl_3{\cdot}6H_2O$ were provided by Cicarelli (Santa Fe, Argentina). Glucose peptone and cetrimide agar were from Britania (Buenos Aires, Argentina). Argon (99.9 purity) was provided by Oxígeno Unión (Río Cuarto, Argentina).

All these compounds were used as received. Water was triply distilled and Buffered KH_2PO_4, $NaHCO_3$, and solutions (each 0.01 M) were employed for pHs/pDs 7 and 9 [23]. In all the cases, pHs/pDs were controlled with a MP220 Mettler-Toledo pH-meter.

2.2. Absorption and fluorescence measurements

Ground state absorption spectra were registered in a Hewlett Packard 8452A diode array spectrophotometer. Steady-state fluorescence was measured with a Spex Fluoromax spectrofluorimeter at 25 ± 1 °C in air-equilibrated solutions. Excitation and emission wavelengths for Rf were 445 and 515 nm, respectively.

2.3. Continuous photolysis

Continuous aerobic photolysis of aqueous solutions containing Rf or RB as photosensitizers plus different substrates were carried out in a PTI (Photon Technology International) unit provided with a high pass monochromator and a 150-W Xe lamp, irradiating at 440 ± 10 nm, or, for non-monochromatic irradiation, in a home-made photolyser with a 150-W quartz-halogen lamp with a cut-off filter (>400 nm).

The reactive rate constant, k_r, for the chemical reaction of $O_2(^1\Delta_g)$ was determined as described in the literature [24], using the expression $\text{slope}/\text{slope}_R = k_r\,[MH]/k_{rR}\,[R]$, for which the knowledge of the reactive rate constant for the photooxidation of a reference compound, R, at similar concentration, is required. The reference R was FFA, with a reported pH-independent k_{rR} value in water of $1.2 \times 10^8\ M^{-1}\ s^{-1}$ [25].

Slope and slope_R are the respective slopes of the first-order plots of oxygen consumption by the substrate and the reference compound, under sensitized irradiation. Oxygen uptake in water was monitored with a 97-08 Orion electrode. Employing RB as a sensitizer, it was assumed that the reaction of $O_2(^1\Delta_g)$ with MH is the only way of oxygen consumption.

2.4. Laser flash photolysis experiments

Argon-saturated aqueous solutions of Rf 0.04 mM were irradiated with a flash photolysis apparatus. A ns Nd:YAG laser system (Spectron) at 355 nm was used for excitation, employing a 150-W Xenon lamp as a source for the analyzing light. The detection system comprised a PTI monochromator and a red-extended photomultiplier (Hamamatsu R666). The signal, acquired and averaged by a digital oscilloscope (Hewlett–Packard 54504A), was transferred via a HPIB parallel interface to a PC where it was analyzed and stored. The disappearance of $^3Rf^*$, a species generated by the 355 nm pulse, was monitored from the first-order decay of the absorbance at 670 nm, a zone where the interference from other possible species is negligible. The decay was measured at low Rf concentration (typically 0.05 mM) and at low enough laser energy to avoid self-quenching and triplet–triplet annihilation.

2.5. Time resolved phosphorescence detection (TRPD) of $O_2(^1\Delta_g)$

The overall quenching rate constant (k_t) for the deactivation of $O_2(^1\Delta_g)$ by MH was determined using a previously reported system [26]. This rate constant is the sum of k_q, the rate constant for physical quenching of $O_2(^1\Delta_g)$ plus the already described k_r. A Nd:YAG laser (Spectron) was employed for the excitation (532 nm) of the sensitizer RB ($Abs_{532} = 0.4$), and the emitted radiation ($O_2(^1\Delta_g)$ phosphorescence at 1270 nm) was detected at right angles using an amplified Judson J16/8Sp germanium detector, after passing through two Wratten filters. The output of the detector was coupled to a digital oscilloscope and to a personal computer for the signal processing. Usually, 16 shots were needed for averaging,

so as to achieve a good signal to noise ratio, from which the decay curve was obtained. Air-saturated solutions were employed in all the cases. In the dynamic determinations, D_2O, instead of H_2O, was used as a solvent in order to enlarge the lifetime of $O_2(^1\Delta_g)$ [25].

2.6. Biodegradation procedure

Ps ATCC 27853 and Bs ATCC 6066, (American Type Culture Collection, Rockville, MD) were used as reference strains. Bacteria were grown in, nutrient broth (nb) supplied by Britania (Buenos Aires, Argentina) and M9 minimal medium (MM) which consists of the following: 3.0 g Na_2HPO_4, 1.5 g KH_2PO_4, 1.0 g NH_4Cl, and 0.5 g NaCl were dissolved in 500 mL distilled water and the pH adjusted to 7.4 with 6 N NaOH. Then the following compounds were added: 0.24 g $MgSO_4$, 0.05 g $CaCl_2{\cdot}6H_2O$, and 0.05 g $FeCl_3{\cdot}6H_2O$.

For the evaluation of the growth rates of Bs and Ps, nb samples were inoculated with a dilution of 1×10^3 CFU/mL (colony-forming units/mL) of a culture of each microorganism in exponential phase with and without added MH solution 0.25 and 0.5 mM and incubated at 32 °C in orbital shaker. The growth rates (h^{-1}) were determined from the slopes of the linear portion of the organism growth curves, which was monitored by measuring the absorbance at 600 nm (A_{600}) and standard plate counts. Planting was carried out in depth in the glucose peptone agar and cetrimide agar for Bs and Ps, respectively, of each nb sample medium at certain time intervals. Plates were incubated during 48 hs at 37 °C. After that period, we performed the count in CFU/mL and such units were plotted versus incubation time.

For Ps, that was able to biodegrade MH in nb, we repeated the growth curve in MM (without carbon and energy sources) supplemented with MH 0.5 mM. The growth curves and growth rates was determined on the conditions previously described. All experiments were done by triplicate in the microbiological and cytostatic Safety Cabinet (Biohazard Class II (BIO-II-A)) under laminar flow. All materials used were previously sterilized.

The biodegradation of MH by Ps was estimated from the growth curve, upon incubation at 32 °C in darkness, in the absence and in the presence of MH by standard plate counts of cetrimide agar. The MH consumption was monitored by the changes absorbance at 328 nm.

The experimental data were processed and analyzed with OriginPro 8 of OrginLab Software Corporation for kinetic analysis. All microbiological experiments were done in triplicate and subjected to statistical analysis of variance (one way ANOVA) with a significance level of $p < 0.05$. These calculations were performed using Statgraphics Centurion XV program. Data were presented as mean value ± standard deviation (SD), which were calculated from triplicate determinations.

3. Results

3.1. General considerations

Scheme 1 depicts the tautomeric and acid–base equilibrium of MH ($pK_a = 5.6$) [27]. B and C correspond to the MH species present at pHs 7 and 9, chosen for the photodegradation studies.

3.2. Stationary photolysis

3.2.1. Riboflavin as a sensitizer in the MH photodegradation

A series of simple experiments were performed in order to roughly evaluate the potential photodegradation of MH under

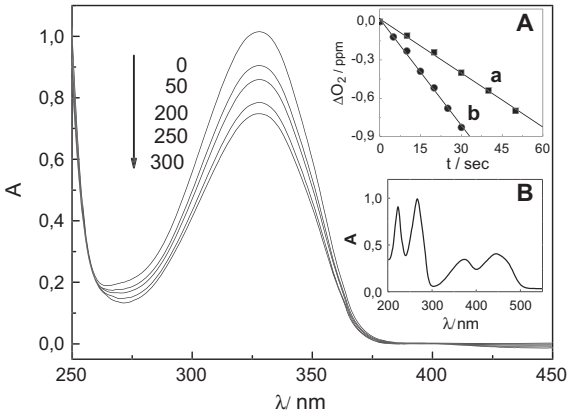

Fig. 1. Changes in the UV–Vis absorption spectrum of a pH 7 aqueous solution of Rf $A_{446} = 0.48$ plus MH 5×10^{-4} M, taken *vs.* Rf $A_{446} = 0.48$, upon visible-light irradiation. Numbers on the spectra represent photoirradiation time in seconds. Inset A: Oxygen consumption *vs.* photoirradiation time for the systems: (a) Rf $A_{446} = 0.40$ plus 0.5 mM MH in aqueous buffer pH 7 and (b) the same in aqueous buffer pH 9, under identical experimental conditions. Inset B: UV–Vis absorption spectrum of 0.04 mM Rf in aqueous buffer pH 7.

Rf-photosensitization. The absorption spectrum of aqueous Rf is shown in Fig. 1.

Air-equilibrated pH 7 and pH 9 aqueous solutions of Rf ($A_{446} = 0.4$) plus 0.5 mM MH were stable when stored under dark conditions. In the presence of light ($\lambda_{irr} > 400$ nm) changes in the MH absorption spectrum were observed, as shown in Fig. 1 for the pH 7 solution. A similar behavior was observed in the pH 9 solution photolized under identical conditions. The photodegradative process was *ca.* 2-times faster for the latter, as estimated from the slopes of their respective graphical representations ΔA_{max}/irradiation time. From parallel experiments on similar photoirradiated solutions, oxygen consumption was observed. Likewise, the oxygen uptake rate (OUR) for the alkaline system, as shown in Fig. 1, inset.

The presence of 0.5 mM of the pesticide in Argon-bubbled Rf solutions, produced a *ca.* 3-fold decrease in the rate of Rf consumption upon photoirradiation. It was evaluated by monitoring the degradation rates of the 445 nm absorption band of Rf in the absence and in the presence of MH. The photodegradation of Rf in deoxygenated solutions is known to occur from the electronically triplet excited state of the vitamin [28].

This preliminary set of experimental facts strongly suggests that under visible light irradiation the overall interaction Rf-MH includes the participation of electronically excited states of the pigment and, according to the oxygen uptake experiments, also involves the participation of dissolved ground state oxygen and/or reactive oxygenated species formed in the medium. Subsequently we carried out a systematic kinetic study in order to analyze and characterize the nature, mechanism and extent of the possible processes implicated in the Rf-sensitized degradation of MH

3.2.2. Quenching of Rf electronically excited states

The fluorescence properties of Rf in water are well known [28]. The presence of MH ⩾ 10 mM produces a detectable quenching of $^1Rf^*$, as determined by stationary emission measurements by monitoring the fluorescence intensity of Rf in the absence (I_o) and in the presence (I) of different MH concentrations, at pH 7. The classical Stern–Volmer treatment ($I_o/I = 1 + {}^1K_{SV}$ [MH], with ${}^1K_{SV} = {}^1k_q \cdot {}^1\tau_o$) allows the determination of the Stern–Volmer constant (${}^1K_{SV} = 20$ - M^{-1}), being ${}^1k_q$ is the rate constant for Rf fluorescence quenching and $\tau_o = 5.2$ ns, the reported value for the fluorescence lifetime of $^1Rf^*$ [29]. A ${}^1k_q$ value of 3.8×10^9 M^{-1} s^{-1} is then deduced.

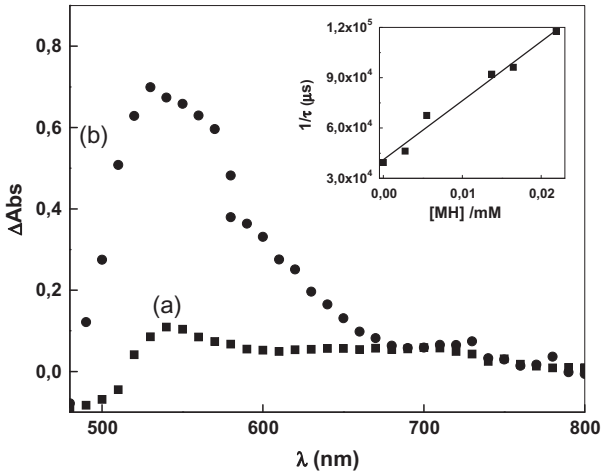

Fig. 2. Transient absorption spectra of Rf (A_{446} = 0.31) in pH 7 argon-saturated aqueous solution, normalized at 700 nm: (a) in the presence of 1 μM MH (b) in the absence of MH, both 2 μs after the laser pulse. Inset: Stern–Volmer plot for the quenching of $^3Rf^*$ by MH. τ refer to $^3Rf^*$ lifetime in the in the presence of different MH concentrations.

The $^3Rf^*$ lifetime in N_2-saturated aqueous solution appreciably decreases in the presence of MH, suggesting the occurrence of an interaction between MH and the mentioned electronically excited state of Rf. As before, a Stern–Volmer treatment of the triplet quenching (Fig. 2, inset), employing the determined $^3Rf^*$ lifetimes in the absence ($^3\tau_o$) and in the presence (τ_o) of MH yielded the bimolecular rate constants $k_{q3} = 3.3 \times 10^9\ M^{-1}\ s^{-1}$ in for the quenching of $^3Rf^*$ in pH 7 aqueous solution.

The transient absorption spectrum of Rf, obtained 2 μs after the laser pulse (Fig. 2, main), is similar to that reported for $^3Rf^*$ in neutral water [30]. Under identical conditions but in the presence of 1 μM of MH (95% $^3Rf^*$ quenched by MH), the shape of the long-lived absorption spectrum is in good agreement with that reported for the neutral Rf radical ($RfH^\cdot$; deprotonation pK_a = 8.3) [31,32].

3.2.3. Interaction of MH with photogenerated ROS

The photosensitized oxidation of MH through ROS was evaluated by means of oxygen consumption experiments, employing specific ROS scavengers. The individual presence of 10 mM NaN_3, 1 μg/mL SOD and 1 μg/mL CAT in air-equilibrated pH 7 aqueous solution of Rf (A_{446} = 0.4) and MH 0.5 mM decreases the OUR, upon photoirradiation (λ_{irr} > 400 nm), as shown in the bars diagram of Fig. 3. Similar experiments with quenchers of ROS have been formerly employed to confirm/discard the participation of $O_2(^1\Delta_g)$, $O_2^{\cdot-}$ and H_2O_2 in a given oxidative event [12, 33, 34]. The salt NaN_3 physically deactivates $O_2(^1\Delta_g)$, whereas the enzyme SOD dismutates the species $O_2^{\cdot-}$ (reaction (1)) and CAT decomposes H_2O_2 (reaction (2)).

$$2O_2^{\cdot-} + 2H^+ + SOD \rightarrow O_2\left(^3\sum_g^-\right) + H_2O_2 \quad (1)$$

$$2H_2O_2 + CAT \rightarrow 2H_2O + O_2\left(^3\sum_g^-\right) \quad (2)$$

3.2.4. Quenching of $O_2(^1\Delta_g)$ by maleic hydrazide. Determination of the rate constants k_t and k_r

TRPD experiments indicates a first order kinetics for the decay of $O_2(^1\Delta_g)$ phosphorescence. The $O_2(^1\Delta_g)$ lifetime agreed well with literature values [25]. The addition of a MH as a quencher lead to a decrease of the $O_2(^1\Delta_g)$ lifetime, unambiguously confirming the interaction of the MH with the oxidative species. The graphically obtained k_t values, as shown in Fig. 4, were $4.8 \pm 0.6 \times 10^8\ M^{-1}\ s^{-1}$ and $3.9 \pm 0.5 \times 10^8\ M^{-1}\ s^{-1}$ at pDs 7 and 9 respectively, employing RB as a dye-sensitizer. The Stern–Volmer treatment $1/\tau\Delta = 1/\tau\Delta_o + k_t$ [MH] was employed, where $\tau\Delta$ and $\tau\Delta_o$ are the $O_2(^1\Delta_g)$ lifetimes in the presence and in the absence MH, respectively.

The rate constants for reactive interaction of the MH were obtained through the already mentioned actinometric method, by monitoring oxygen photoconsumption, employing FFA as a reference compound and RB (A_{530} = 0.4) as a dye sensitizer. The values $k_r = 1.5 \pm 0.3 \times 10^7\ M^{-1}\ s^{-1}$ and $1.2 \pm 0.3 \times 10^8\ M^{-1}\ s^{-1}$ at pHs 7 and 9 respectively were graphically determined from the first order plots shown in Fig. 4.

3.2.5. Humic acid as a photosensitizer

Photoirradiation of individual solutions of 0.5 mM MH plus Rf (A_{446} = 0.5) and 0.5 mM MH plus HA (A_{380}) = 0.38 produces oxygen consumption (cut-off filter λ > 400 nm). The OUR in the solution photosensitized by the vitamin was *ca.* 50 times faster than that of the HA-photosensitized one, as shown in Fig 5 main. On the other hand, as shown in Fig. 5 inset A, the OUR by a solution

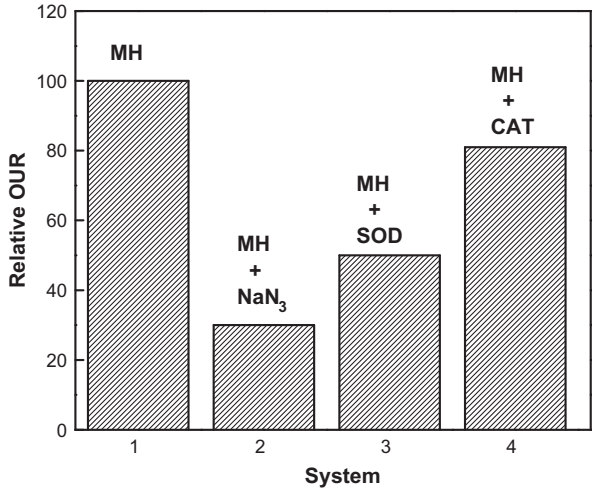

Fig. 3. Relative values of oxygen uptake rates (OUR) for 0.5 mM MH + Rf (A_{446} = 0.4) as a function of photoirradiation time (cut-off 400 nm) in aqueous pH 7 buffer solution, in the absence (1) and in the presence of 0.9 mM NaN_3 (2); 1 μg/mL SOD (3) and 1 μg/mL CAT (4).

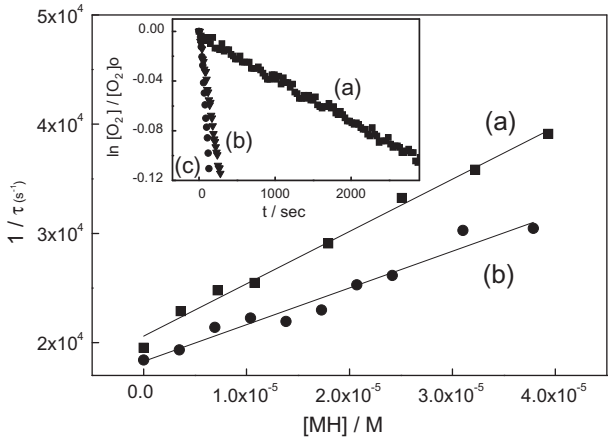

Fig. 4. Stern–Volmer plots for the quenching of $O_2(^1\Delta_g)$ phosphorescence by MH. Photosensitizer: RB (A_{532} = 0.3) in pH 7 (a) and pH 9 (b) aqueous solutions. Inset: First order plots of Oxygen consumption *vs.* photoirradiation time for the systems RB (A_{560} = 0.48) plus: 0.5 mM MH pH 7 (a); 0.5 mM MH pH 9 (b) and 0.5 mM FFA pH 7 (c).

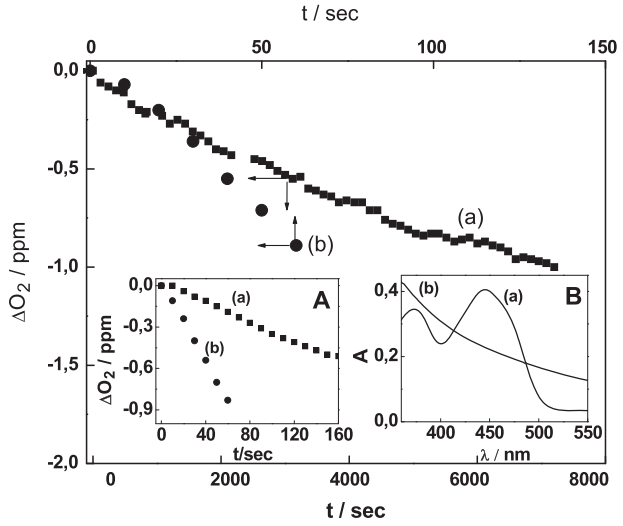

Fig. 5. Oxygen consumption *vs.* photoirradiation time in pH 7 buffer aqueous solutions for the systems: HA (A_{400} = 0.32) plus 0.5 mM MH (a) and Rf (A_{446} = 0.42) plus 0.5 mM MH (b). Inset A: Oxygen consumption *vs.* photoirradiation time in pH 7 aqueous solutions for the systems: HA (A_{400} = 0.32) plus Rf (A_{446} = 0.42) plus 0.5 mM MH (a) and Rf (A_{446} = 0.42) plus 0.5 mM MH (b). Inset B: Absorption spectra of Rf (a) and HA (b) in pH 7 aqueous solution.

0.5 mM MH is reduced in *ca.* 5 times employing the sensitizing mixture Rf (A_{446} = 0.5) plus HA (A_{380} = 0.38), as compared to that obtained employing the vitamin (A_{446} = 0.5) as the only photosensitizer. The photoirradiation conditions were identical in all the described experiments. Besides, no oxygen uptake at all was observed upon photolysis of individual solutions of 0.5 mM MH, HA (A_{380} = 0.38) and Rf (A_{446} = 0.5), the latter within the photoirradiation time employed for the corresponding run in the presence of MH. Direct photodecomposition of MH due to direct light absorption can be disregarded in the presence of the mentioned cut-off filter. These pieces of experimental evidence strongly suggest that the observed oxygen consumption operates through a non simple HA- and Rf-photosensitization mechanism in addition to the mutual inner filter effect. The respective shapes of the absorption spectra of Rf and HA are shown in Fig. 5, inset B.

3.2.6. The photodegradation rate of MH as compared to known contaminant/biological photooxidizable targets

Taking the OUR as a global estimation of the photooxidation rate and in order to achieve a realistic estimation of the environmental fate of MH under Rf-sensitized photoirradiation with visible light, the OUR of the pesticide was compared to those of individual ph and trp under identical conditions. The relative values for OUR were 1 for trp, 0.44 for MH and 0.18 for ph. Phenol and trp represent classic examples of contaminant and biological photooxidizable targets, and their respective kinetic and mechanistic aspects of Rf-photoinduced degradation are well known: Both ph and trp are oxidized by $O_2(^1\Delta_g)$ and $O_2^{\cdot-}$ produced in the photosensitization process. Type-I (radical-mediated) and specially Type II ($O_2(^1\Delta_g)$-mediated) are the main reported mechanisms responsible for Rf-photosensitized degradation of trp. An exclusive Type I mechanism operates in the case of ph at pH7 [35,36,37].

3.2.7. Microbiological assays

The presence of MH in concentration 0.25 and 0.5 mM in Ps and Bs cultures cause noticeable changes in the respective growth rate of the bacteria.

MH produces a reduction of the growth rate of Bs from 1.61 ± 0.08 (h^{-1}) in the absence of MH to 0.96 ± 0.05 (h^{-1}) and 0.67 ± 0.03 (h^{-1}), in the presence of the pesticide at the respective mentioned concentrations (Fig. 6A).

The case of Ps is quite different. An increase in the growth rate from 0.60 ± 0.02 (h^{-1}) in the absence of MH to 0.70 ± 0.02 (h^{-1}) and 0.75 ± 0.02 (h^{-1}) in the presence of 0.25 and 0.50 mM of the pesticide respectively can be observed from the data in Fig. 6B.

Fig. 7 shows the increase in the growth rate of Ps in MM, in the presence of 0.4 mM MH (main figure). The growth of Ps is concomitantly corresponded by a biodegradation of the herbicide, as a function of the incubation time (Fig. 7, inset). From the MH consumption rates a MH half-live of 250 ± 15 h was estimated.

4. Discussion

4.1. Sensitized photodegradation of MH

The observed decrease in the rates of oxygen uptake in the presence of selective ROS scavengers indicates the participation of $O_2(^1\Delta_g)$, $O_2^{\cdot-}$ and H_2O_2 in the overall photosensitized oxidative event of MH.

The species $O_2(^1\Delta_g)$ is produced by energy transfer from $^3Rf^*$ to dissolved ground state oxygen [25], whereas it is well known that the species $O_2^{\cdot-}$ and H_2O_2 are produced from $^3Rf^*$ after a series of electron transfer reactions in the presence of an electron-donor species, represented by MH in this case [1,31,36].

The quenching of $O_2(^1\Delta_g)$ by MH was unambiguously demonstrated by the TRPD experiments. The respective k_t values at pHs 7 and 9 are very close and practically coincident within the experimental error. Nevertheless, the reactive rate constants k_r are quite different at both pH values, following the typical behavior expected for N-heteroaromatic hydroxyl derivatives [1], a structural moiety present in the MH molecule. The OH-ionized group greatly enhances the effective $O_2(^1\Delta_g)$-mediated photooxidation [38]. According to Scheme 1 and to the pK value for MH, *ca.* 20% of the hydroxyl group would be in the molecular form at pH 7 whereas the molecule will be totally ionized at pH 9. In parallel, the tautomeric quinonic form A (Scheme 1), present at pH 7, only would contribute to $O_2(^1\Delta_g)$ physical quenching as already reported by ourselves for a series of *p*-quinonic compounds [39].

The $O_2(^1\Delta_g)$-mediated photooxidation quantum efficiency φ_r ($\varphi_r = k_r[Q]/(k_d + k_t[Q])$) is not easy to evaluate, particularly in natural environments, because its determination includes the knowledge of the actual concentration of the photooxidizable substrate [38]. A simpler and useful approach is the determination of the k_r/k_t ratio, which indicates the fraction of overall quenching of $O_2(^1\Delta_g)$ by the substrate that effectively leads to a chemical transformation. k_r/k_t ratios for MH at pHs 7 and 9 respectively are 0.03 and 0.30.This result indicates that the $O_2(^1\Delta_g)$-mediated degradation of MH efficiently operates in the alkaline range whereas is rather poor at pH values closer to the environmental ones.

The simultaneous presence of HA and Rf as daylight photosensitizers could represent the case of superficial layers in lakes and water courses, where the concentration of the sensitizers is not sufficient to reach optically dense conditions. In the present case, the photosensitizing system HA + Rf fairly reduces the Rf-mediated MH oxidative rate, as estimated by oxygen consumption experiments (Fig. 5). The vitamin possesses a quantum yield for $O_2(^1\Delta_g)$ ten times higher than the corresponding one for HA and the overall sensitizing effect still operating, mainly due to the Rf contribution. It has been reported by ourselves that the mixture HA + Rf generates $O_2(^1\Delta_g)$ and $O_2^{\cdot-}$, the latter especially in the presence of electron donors such as hydorxyaromatic compounds [40]. Besides, HA constitutes a relatively labile photosensitizer. Its

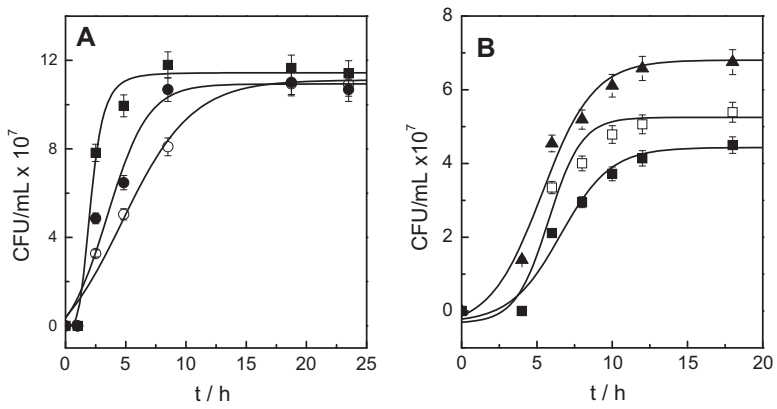

Fig. 6. A: Growth curve of *Bacillus subtilis* in the absence of MH (■) and in the presence 0.25 mM MH (●) and 0.5 mM MH (○). B: Growth curve of *Pseudomonas aeruginosa* in the absence of MH (■) and and in the presence of 0.25 mM MH (▲) and 0. 5 mM MH (□). Both experiments at 32 °C in nb.

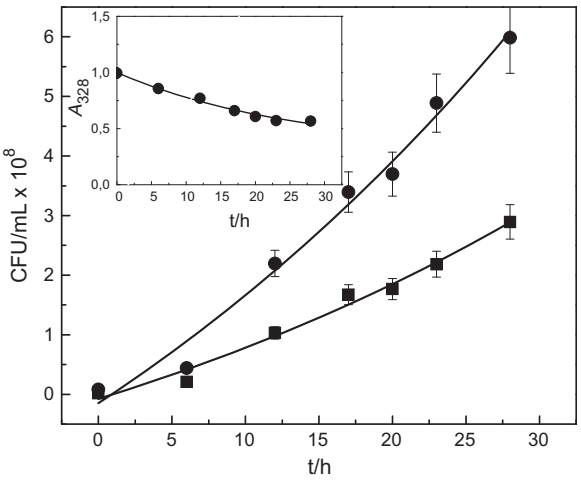

Fig. 7. Growth curve of *Pseudomonas aeruginosa* in MM incubated at 32 °C, in the absence (■) and in the presence of 0.4 mM MH (●). Inset: Absorbance decrease of 0.4 mM MH monitored at 328 nm *vs.* incubation time in the presence of *Pseudomonas aeruginosa* incubated in MM at 32 °C.

autoxidation, mediated by $O_2(^1\Delta_g)$, has been mentioned as one of the possible pathways for the self-degradation in natural waters [13]. Other HA degradation routes have been reported, including the $O_2^{\cdot-}$-catalytic decomposition [41,42]. In the present case this channel should be particularly active after the interaction MH-^{3}Rf*.

The overall rate of oxygen uptake by individual ph, trp and MH solutions upon Rf-sensitization can be considered as a direct measure of substrate photooxidability, for comparative purposes. Results indicate, in kinetic terms, that all three compounds are photodecomposed within a factor 4 in their respective rate values, with MH in an intermediate position.

4.2. Microbiological effect on MH

Our experimental evidence clearly indicates that MH exerts a bacteriostatic effect on Bs, whereas Ps is able to degrade the pesticide.

According to our knowledge, MH biodegradation was not previously studied. The only work found in literature that reports on this topic was carried out by Ingerslev and Nyholm [43], who reported the biodegradation rate in surface water systems of 14*C*-labeled compounds, among which are some herbicides such as atrazine and MH. In that study the biodegradation was caused by the native microbiota of the different water samples analyzed. The authors found differences in the biodegradation rate, which depend mainly on the type of compound and origin of the water sample. In particular MH presents a relatively low rate of biodegradation but higher than that of other herbicides such as atrazine. As this study was done on surface water, it is very likely the presence of Ps in the native microbiota [17].

MH bacteriostatic effect on Bs, has not been previously reported. Shiau et al. [44], evaluated the mutagenic effect and damage on DNA of Bs produced by a series of pesticides, including MH. The authors observed that MH exerted a marked mutagenic effect and a weak activity of DNA damage, possibly associated to the antibacterial effect presented by the herbicide.

In our work, the MH biodegradation by Ps was estimated by the increase in the growth rate of the microorganism in the presence of MH, and by MH consumption.

The experiments in MM (Fig. 7), where MH is the only source of carbon [45], strongly suggest that Ps uses the herbicide as a carbon and energy source.

5. Conclusions

The herbicide MH is effectively degraded by the conjunctive action of photogenerated ROS such as $O_2(^1\Delta_g)$, $O_2^{\cdot-}$ and H_2O_2 and the bacteria Ps. The simultaneous presence of the visible light-absorber Rf, HA and the aquatic microorganism intend to mimic a natural MH contaminated environment. The rate of the degradation photoprocess is comparable with that of ph, profusely employed as a contaminant model.

6. Abbreviations

ANOVA	analysis of variance
ATCC	American Type Culture Collection
Bs	bacillus subtilis
CAT	catalase
CFU	colony-forming units
D_2O	deuterium oxide
DNA	deoxyribonucleic acid
FFA	furfuryl alcohol
HA	humic acid
H_2O_2	hydrogen peroxide
MH	maleic hydrazide
MM	minimal medium
NaN_3	Sodium azide

(*continued on next page*)

nb	nutrient broth
$O_2^{\cdot-}$	superoxide radical anion
$O_2(^1\Delta_g)$	singlet molecular oxygen
OUR	oxygen uptake rates
ph	phenol
Ps	pseudomonas aeruginosa
PTI	Photon Technology International
R	reference
RB	Rose Bengal
Rf	riboflavin
ROS	reactive oxygen species
SOD	superoxide dismutase
Trp	L-tryptophan
TRPD	time resolved phosphorescence detection

Acknowledgements

Financial support from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Ministerio de Ciencia y Tecnología de Córdoba, Secretarías de Ciencia y Técnica of the Universidad Nacional de Río Cuarto (SECyT UNRC), of the Universidad Nacional de la Patagonia SJB (SECyT UNP-SJB), of the Universidad Nacional de la Patagonia Austral (SECyT UNPA) and of the Universidad Tecnológica Nacional (SECyT UTN), all from Argentine, is gratefully acknowledged.

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