Toxicology in Vitro xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Toxicity assessment of the herbicide metolachlor comparative effects on bacterial and mitochondrial model systems

Susana P. Pereira [a], Maria A.S. Fernandes [b], João D. Martins [c], Maria S. Santos [a], António J.M. Moreno [b], Joaquim A.F. Vicente [d], Romeu A. Videira [e], Amália S. Jurado [c,*]

[a] CNC – Centre for Neuroscience and Cell Biology, Zoology Department, Coimbra University, Portugal
[b] IMAR, Zoology Department, Coimbra University, Portugal
[c] CNC, Biochemistry Department, Coimbra University, Portugal
[d] IMAR, Botany Department, Coimbra University, Portugal
[e] ESTV, Politechnique Institut, Viseu, Portugal

ARTICLE INFO

Article history:
Received 3 November 2008
Accepted 30 June 2009
Available online xxxx

Keywords:
Herbicides
Metolachlor
Mitochondria
Bacillus stearothermophilus
Toxicity assessment

ABSTRACT

Metolachlor is one of the most intensively used chloroacetamide herbicides. However, its effects on the environment and on non-target animals and humans as well as its interference at a cell/molecular level have not yet been fully elucidated. The aim of this study was: firstly, to evaluate the potential toxicity of metolachlor at a cell/subcellular level by using two *in vitro* biological model systems (a strain of *Bacillus stearothermophilus* and rat liver mitochondria); secondly, to evaluate the relative sensibility of these models to xenobiotics to reinforce their suitability for pollutant toxicity assessment. Our results show that metolachlor inhibits growth and impairs the respiratory activity of *B. stearothermophilus* at concentrations two to three orders of magnitude higher than those at which bacterial cells are affected by other pesticides. Also at concentrations significantly higher than those of other pesticides, metolachlor depressed the respiratory control ratio, membrane potential and respiration of rat liver mitochondria when malate/glutamate or succinate were used as respiratory substrates. Moreover, metolachlor impaired the respiratory activity of rat liver mitochondria in the same concentration range at which it inhibited bacterial respiratory system (0.4–5.0 μmol/mg of protein). In conclusion, the high concentration range at which metolachlor induces toxicity *in vitro* suggests that this compound is safer than other pesticides previously studied in our laboratory, using the same model systems. The good parallelism between metolachlor effects on both models and the toxicity data described in the literature, together with results obtained in our laboratory with other compounds, indicate the suitability of these systems to assess toxicity *in vitro*.

1. Introduction

Worldwide pesticide usage has dramatically increased during the last decades, coinciding with changes in farming practices and the increasingly intensive agriculture. This widespread use of pesticides (65% being herbicides, Stevens et al., 1991) for agricultural and non-agricultural purposes has resulted in the presence of their residues in various environmental matrices. The presence of pesticides in soil and water systems has been well documented worldwide (Huber et al., 2000; Cerejeira et al., 2003) and can cause serious problems both to the environment and to human health (Koeman, 1984). Metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide], discovered in 1974 by Ciba-Geigy Co., Basel Switzerland (Kontchou and Gschwind, 1998), is the most intensively used herbicide in the chemical class of chloroacetamides (see Fig. 1). It is considered a hydrophobic herbicide, with relatively low solubility in water (530 mg/l at 20 °C) and high lipophilicity, characterized by its partition coefficient between octanol/water of 2.9–3.3 at 25 °C (Miller et al., 1997; Weber et al., 2006). Due to a relatively high stability in aqueous media, the ability for leaching and a low rate of degradation (Miller et al., 1997; Accinelli et al., 2006), metolachlor is a potential contaminant of water networks (Aguilar et al., 1998; Albanis et al., 1998).

Microorganisms are often used as indicators of soil health and constitute good tools for toxicological characterization of pollutants (Nielsen and Winding, 2002). In our laboratory, a strain of the thermophilic Gram-positive eubacterium *Bacillus stearothermophilus* has been used as a predictive model for assessing the toxicity of different compounds. Toxicity tests using *B. stearothermophilus* have allowed to establishing toxicity correlations for insecticides and organometals, regarding effects on energy metabolism and cell

* Corresponding author. Address: Biochemistry Department, Coimbra University, Apartado 3126, 3001-401 Coimbra, Portugal. Tel.: +351 239 853600; fax: +351 239 853607.
E-mail address: asjurado@bioq.uc.pt (A.S. Jurado).

0887-2333/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2009.06.032

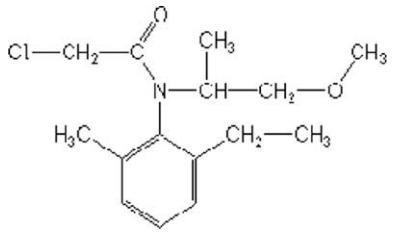

Fig. 1. Chemical structure of metolachlor.

viability (Donato et al., 1997; Martins et al., 2003, 2005; Monteiro et al., 2003). On the other hand, mitochondria are recognized as a suitable model to evaluate *in vitro* toxicological actions of several xenobiotics, providing data well correlated with toxicity parameters derived from cell cultures and whole organisms (Eisenbrand et al., 2002; Bragadin, 2006). Because of their central role in cell life and death, as ATP producers, regulators of intracellular Ca^{2+} homeostasis and generators of intracellular ROS (Duchen, 2004; Cardoso et al., 2008), mitochondria constitute an important cellular target for xenobiotic toxic action (Starkov and Wallace, 2002; Scatena et al., 2007; Pereira et al., 2009).

In the present work, we evaluated the potential toxicity of metolachlor by using *B. stearothermophilus* and rat liver mitochondria as biological model systems. These *in vitro* models were chosen because they have successfully been used for studying the toxicity of a wide number of pollutants from the whole organism to the molecular level. Data provided by both model systems will be compared to evaluate their relative sensibility to xenobiotics and to highlight their suitability for chemical toxicity screening.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA). Metolachlor 98.2%, dissolved in absolute dimethyl sulfoxide (DMSO), was stored at −20 °C, in hermetically sealed glass tubes. At this temperature, precipitation is not observed and evaporation is minimized. In all experimental procedures, reagents of high purity (⩾95%) were used.

2.2. Bacterial cultures

The strain of *B. stearothermophilus* was supplied by Mast Laboratories, UK (lot #7953). The conditions of its maintenance and growth have been previously described (Jurado et al., 1987). Liquid cultures were started with an early stationary phase inoculum and grown in 1 L Erlenmeyer flasks containing 200 ml of a complex growth medium (L-Broth), shaken at 130 rpm in a New Brunswick water bath shaker, at 65 °C. Metolachlor was added to the growth medium from a concentrated solution in order to obtain concentrations ranging from 100 to 600 μM. Control cultures were grown in media without herbicide, but with a volume of DMSO corresponding to the maximum amount of metolachlor solution assayed in the growth experiment. Growth was monitored by measuring the turbidity at 610 nm, in a Bausch and Lomb Spectronic 21 spectrophotometer.

2.3. Animals

Male Wistar rats (250–350 g), housed at 22 ± 2 °C under artificial light for 12-h light/dark cycle and with access to water and food *ad libitum*, were used throughout the experiments, carried out in accordance with the National Requirements for Vertebrate Animal Research and the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes.

2.4. Isolation of bacterial protoplasts

Protoplasts were prepared from bacterial cultures harvested in the middle of the exponential growth phase, by incubation with lysozyme, essentially as described by Wisdom and Welker (1973). The protein content of the protoplasts was determined by the biuret method, calibrated with bovine serum albumin (Gornall et al., 1949).

2.5. Isolation of rat liver mitochondria

Rat liver mitochondria were isolated from male Wistar rats (6 weeks) by differential centrifugation according to conventional methods (Gazotti et al., 1979). After washing, the pellet was gently resuspended in the washing medium (250 mM sucrose and 10 mM HEPES-KOH, pH 7.2) at a protein concentration of about 50 mg ml^{-1}. Protein content was determined by the biuret method (Gornall et al., 1949).

2.6. Measurement of bacterial oxygen consumption

The respiratory activity of the protoplasts was monitored polarographically with a Clark oxygen electrode (Estabrook, 1967) connected to a recorder, in a thermostated water-jacketed closed chamber (1 ml), with magnetic stirring, at 40 °C. Aliquots of a concentrated metolachlor solution were added to the reaction medium (40 mM HEPES, pH 7.5) containing the protoplasts (0.5 mg of protein), and each preparation was incubated for 30 min, before the addition of the respiratory substrate. The effects of metolachlor on oxygen consumption of protoplasts were examined using 10 mM NADH or 10 mM ascorbate plus 600 μM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) as respiratory substrates. For each metolachlor concentration assayed, a control was prepared containing an equivalent volume of the solvent (DMSO). Oxygen consumption rate in the presence of the herbicide was expressed as % of the control (0 μM metolachlor).

2.7. Measurement of mitochondria oxygen consumption

Mitochondria oxygen consumption was monitored at 30 °C with a Clark-type electrode, using the same system previously described for protoplasts. Mitochondria (1.5 mg protein) were incubated for 3 min in 1.5 ml of a medium containing 250 mM sucrose, 10 mM Hepes-KOH (pH 7.2), 20 mM KCl, 5 mM K_2HPO_4, and 2 mM $MgCl_2$, supplemented with 2 μM rotenone and different metolachlor concentrations, before energization with 10 mM malate/5 mM glutamate or 10 mM succinate. To induce state 3 respiration, adenosine diphosphate (ADP, 200 μM) was added. Uncoupled respiration was initiated by the addition of 1 μM *p*-trifluoromethoxyphenylhydrazone (FCCP). O_2 consumption was calculated considering that the saturation oxygen concentration was 232 nmol O_2 per ml of reaction medium at 30 °C. Control values are expressed in nmol O_2 mg^{-1} protein min^{-1}. The respiratory control ratio (RCR) and ADP to oxygen ratio (ADP/O) were calculated according with previously described methods (Chance and Williams, 1956).

2.8. Measurement of mitochondrial transmembrane potential ($\Delta\psi$)

The mitochondrial transmembrane potential ($\Delta\psi$) was measured indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+-selective electrode, as previously described (Kamo et al., 1979). Mitochondria (1.5 mg

Please cite this article in press as: Pereira, S.P., et al. Toxicity assessment of the herbicide metolachlor comparative effects on bacterial and mitochondrial model systems. Toxicol. In Vitro (2009), doi:10.1016/j.tiv.2009.06.032

protein) were incubated for 3.5 min at 30 °C in 1.5 ml of medium containing 250 mM sucrose, 10 mM Hepes-KOH (pH 7.2), 20 mM KCl, 5 mM K_2HPO_4, and 2 mM $MgCl_2$, supplemented with 3 μM TPP^+. Energization was started by adding 10 mM malate plus 5 mM glutamate or 10 mM succinate plus 2 μM rotenone or 5 mM ascorbate plus 0.25 mM TMPD. No correction was made for the "passive" binding of TPP^+ to the mitochondria membranes because the purpose of the experiments was to show relative changes in transmembrane potential rather than absolute values. As a consequence, we can anticipate some overestimation for the $\Delta\psi$ values. Metolachlor did not affect TPP^+ binding to mitochondria membranes or the electrode response.

2.9. Statistical analysis

All the experiments were performed using three or more independent experiments with different preparations. The values are expressed as mean ± standard deviation. Means were compared using ANOVA with the Student–Newman–Keuls as a post-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of metolachlor on the bacterial growth

A strain of *B. stearothermophilus*, currently used in our laboratory as a model for studying the cytotoxicity of different groups of pesticides, was grown in the optimal temperature range (65 °C) in a complex medium (dilute L-Broth) to which metolachlor from a concentrated solution was added to obtain concentrations ranging from 100 to 600 μM. As a control, a culture was grown in a medium without herbicide but with a volume of DMSO corresponding to the maximal amount of metolachlor solution assayed in the growth experiment (see Fig. 1).

The addition of increasing concentrations of metolachlor to the growth medium induced progressively lower specific growth rates and bacterial yields, as documented in Table 1. Growth in the presence of 500 μM metolachlor reduced the specific growth rate by approximately 50% as compared to the control culture and the cell yield decayed about 64%. The lag time was also affected by the herbicide. In the presence of 600 μM metolachlor, the length of the lag period was about 10 times higher than that of the control. Additionally, a very limited growth occurred in these conditions. Plotting the growth parameters documented in Table 1 as function of the pesticide concentration (data not shown), the concentrations at which metolachlor increased the lag period, or reduced the bacterial yield and the specific growth rate by 50% relatively to the control (EC_{50}) were determined by interpolation, ranging 400 to about 500 μM (Table 1).

3.2. Effects of metolachlor on the oxygen consumption of bacterial protoplasts

Protoplasts prepared from cells of *B. stearothermophilus* grown in the basal medium, in the optimal temperature range (65 °C), were used to assess the effect of metolachlor on the oxygen consumption rate measured at 40 °C. Protoplasts were incubated with metolachlor in the concentration range of 0.5–5.0 μmol/mg of protein, during a period of 30 min prior to the addition of the respiratory substrate. The NADH-supported oxygen consumption rate, expressed in percentage of the control (protoplasts incubated with a volume of DMSO corresponding to the maximum amount of metolachlor solution assayed in the respiration experiment), showed a progressive decrease with increasing concentrations of the herbicide, as documented in Fig. 2 and Table 2. At a concentration of 1.2 μmol/mg of protein, metolachlor reduces the oxygen consumption by 50% relatively to the control, as determined by interpolation of a plot of the oxygen consumption rate as function of the herbicide concentration.

When a mixture of ascorbate + TMPD was used instead of NADH as respiratory substrate, any significant effect was noticed on the respiratory activity of the protoplasts, in the same metolachlor concentration range (data not shown). As expected, the oxygen consumption supported by NADH or ascorbate + TMPD was completely impaired by the addition of KCN (1 mM), as a consequence of the complete inhibition of the terminal oxidase (Fig. 2).

3.3. Effects of metolachlor on mitochondrial bioenergetics

The effects of metolachlor on malate/glutamate- and succinate-supported respiratory rates (state 4 and state 3) of rat liver mitochondria are depicted in Fig. 3A. State 4 respiration was progressively stimulated whereas state 3 respiration was inhibited by increasing concentrations of metolachlor either using malate/glutamate (reducing Complex I) or succinate (reducing Complex II) as respiratory substrates. In the presence of 800 nmol metalochlor/mg of protein, 22% stimulation of state 4 respiration and 44% inhibition of state 3 respiration were noticed with malate + glutamate as substrate and 40% stimulation of state 4 and 36% inhibition of state 3 respiration, for succinate-supported respiration. The FCCP-uncoupled respiration supported by succinate was also significantly inhibited (40%) by 800 nmol metalochlor /mg of protein (data not shown).

Fig. 3B shows that metolachlor, in the concentration range of 400–1000 nmol/mg of protein, significantly depressed RCR (from about five in the absence of herbicide, using DMSO as vehicle control, to less than two) with both respiratory substrates. The ADP/O ratio was also decreased by metolachlor, in the same concentration range, from about 3 (without herbicide) to 2.2 when respiration

Table 1
Lag time, specific growth rate and final cell density of cultures of *B. stearothermophilus* grown in a basal medium (L-Broth) without (control) or with different concentrations of metolachlor. EC_{50} in μM for the different growth parameters are also shown.

Metolachlor (μM)	Lag time (h)[a]	Specific growth rate (h^{-1})[a]	Final cell density (% of control)[a]
0	0.12 ± 0.03	2.48 ± 0.16	100
100	0.18 ± 0.02$^{n.s}$	2.26 ± 0.14$^{n.s}$	100 ± 1.31$^{n.s}$
200	0.23 ± 0.02$^{n.s}$	2.08 ± 0.18$^{n.s}$	72.77 ± 2.77$^{n.s}$
300	0.34 ± 0.07$^{n.s}$	1.87 ± 0.31*	69.89 ± 2.41***
400	0.56 ± 0.07***	1.47 ± 0.24***	47.63 ± 4.56$^{***,°°°}$
500	1.03 ± 0.12$^{***,°°°}$	1.28 ± 0.15***	35.83 ± 6.82***
600	1.12 ± 0.01***	0.4849 ± 0.13$^{***,°}$	28.21 ± 1.04***
EC_{50} (μM)	408	463	406

[a] Values of lag time, specific growth rates and final cell densities are means ± standard deviation from three or more independent experiments and comparisons were performed using one way ANOVA, with the Student–Newman–Keuls as a post-test, for the following paired observations: control culture (0 μM metolachlor) vs. cultures grown with different concentrations of metolachlor (n.s., not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$); cultures grown with different metolachlor concentrations vs. culture with the concentration immediately below (n.s., not significant; $^{°}p < 0.05$; $^{°°}p < 0.01$; $^{°°°}p < 0.001$).

Please cite this article in press as: Pereira, S.P., et al. Toxicity assessment of the herbicide metolachlor comparative effects on bacterial and mitochondrial model systems. Toxicol. In Vitro (2009), doi:10.1016/j.tiv.2009.06.032

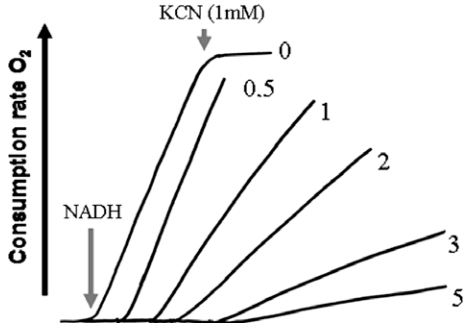

Fig. 2. Effects of metolachlor in the oxygen consumption of *B. stearothermophilus* protoplasts supported by 10 mM NADH. Concentrations of the herbicide (μmol/mg protein) and of KCN are displayed in the figure.

Table 2
Effects of metolachlor on the oxygen consumption rate of *B. stearothermophilus* protoplasts using NADH as substrate, expressed as % of the control. EC_{50} in μmol/mg protein is also shown.

Metolachlor (μmol/mg protein)	O_2 consumption rate (% of the control)[a]
0.00	100.00
0.50	73.19 ± 4.00***
1.00	49.44 ± 1.51$^{***,\circ\circ\circ}$
2.00	28.83 ± 1.83$^{***,\circ\circ\circ}$
3.00	19.85 ± 1.40***
5.00	8.97 ± 0.20$^{***,\circ}$
EC_{50} (μmol/mg protein)	1.20

[a] Results are means ± standard deviation of at least three independent experiments and comparisons were performed using one way ANOVA, with the Student–Newman–Keuls as a post-test, for the following paired observations: protoplasts incubated with different concentrations of metolachlor vs. control protoplasts (n.s., not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$) or protoplasts incubated with different concentrations of metolachlor vs. protoplasts with the concentration immediately below (n.s., not significant; $^{\circ}p < 0.05$; $^{\circ\circ}p < 0.01$; $^{\circ\circ\circ}p < 0.001$).

was supported by malate/glutamate and from about 2 to 1.2, when succinate was used as substrate.

The effect of metolachlor on the mitochondrial membrane potential ($\Delta\psi$) was also studied (Fig. 3C and Table 3). Mitochondria in the control conditions (in the absence of herbicide and using DMSO as vehicle control) developed a $\Delta\psi$ between 210 and 215 mV (negative inside) upon substrate addition. A progressive decrease of the potential was observed upon incubation with increasing concentrations of metolachlor (from 400 to 1000 nmol/mg of protein). This alteration showed the same magnitude when malate/glutamate or succinate were used as the respiratory substrate (e.g. a decrease of 7% in the presence of 800 nmol metolachlor/mg of protein). Metolachlor also depressed the phosphorylation rate for both substrates (Fig. 3C). A depression of about 40% was observed in the presence of 800 nmol metolachlor /mg of protein.

4. Discussion

Metolachlor is one of the most intensively used chloroacetamide herbicides. Although generally considered a relatively safe pesticide, a full elucidation of its environmental effects as well as its action on non-target animals and humans is missing. In the present work, we studied the potential toxicity of metolachlor by using two *in vitro* models (a strain of *B. stearothermophilus* and rat liver mitochondria), which have provided toxicological parameters (Donato et al., 1997; Eisenbrand et al., 2002; Martins et al., 2005; Fernandes et al., 2008) well correlated with toxicity indices (LD_{50} or LC_{50}) for mammals or fishes.

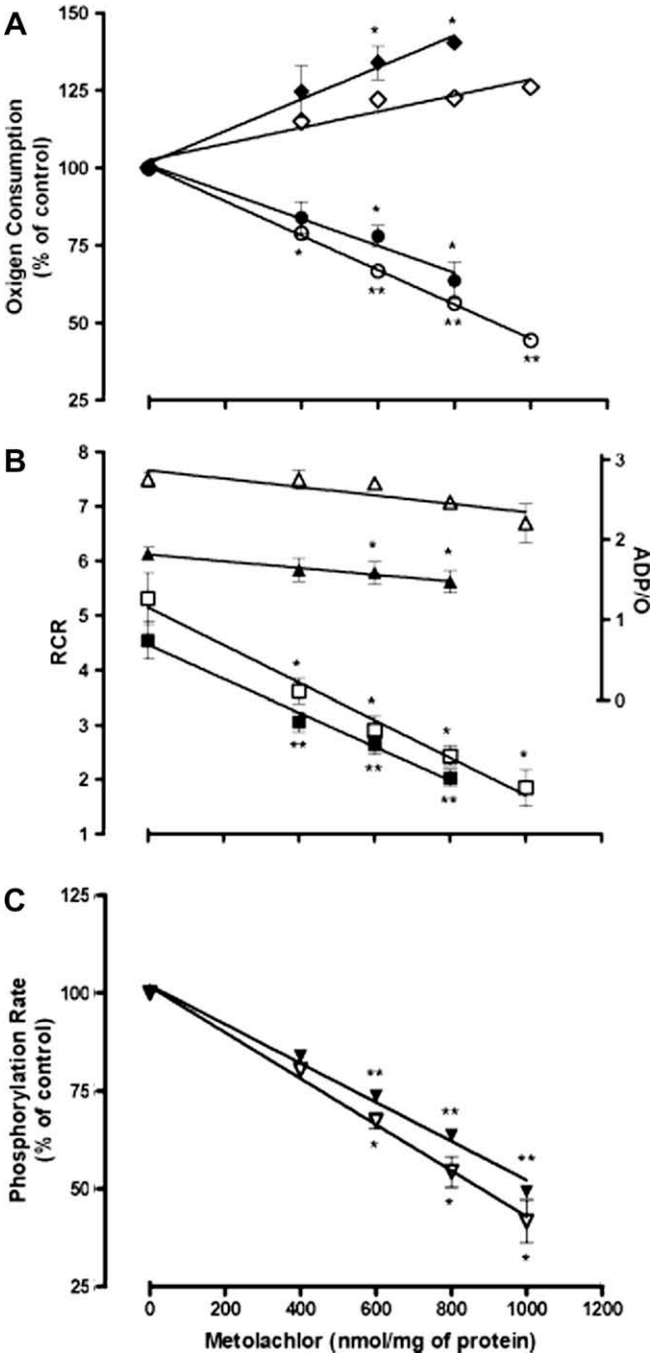

Fig. 3. Effects of metolachlor on malate/glutamate- and succinate-supported respiratory activity and phosphorylation rate of rat liver mitochondria. (A) Comparison of metolachlor effects on malate/glutamate- (open circles) and succinate-supported (filled circles) O_2 consumption in state 4 (◇, ♦) and in state 3 (○, ●) respiration. Control values expressed in nmol O_2 mg^{-1} protein min^{-1}: state 4 = 4.64 ± 0.38 or 8.12 ± 0.77; state 3 = 24.803 ± 3.8 or 36.8 ± 4.5 for malate/glutamate- or succinate-supported respiration, respectively. The results correspond to the mean ± standard error of the mean of three experiments obtained from different mitochondrial preparations. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. control (DMSO). (B) Comparison of metolachlor effects on the respiratory indices RCR (□, ■), and ADP/O (Δ, ▲), using malate/glutamate – (open symbols) or succinate (filled symbols) as respiratory substrate. The results correspond to the mean ± standard error of three experiments obtained from different mitochondrial preparations. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. control (DMSO). (C) Comparison of metolachlor effects on malate/glutamate – (▽) and succinate-dependent (▼) phosphorylation rates. Control values expressed in nmol ADP mg $protein^{-1}$ min^{-1}: malate/glutamate = 115.7 ± 5.20; succinate = 114.67 ± 5.34. The results correspond to the mean ± standard error mean of three experiments obtained from different mitochondrial preparations.

Please cite this article in press as: Pereira, S.P., et al. Toxicity assessment of the herbicide metolachlor comparative effects on bacterial and mitochondrial model systems. Toxicol. In Vitro (2009), doi:10.1016/j.tiv.2009.06.032

Table 3
Effects of metolachlor on the transmembrane potential ($\Delta\Psi$ of rat liver mitochondria, in a concentration range of 0–1000 nmol/mg protein.

Metolachlor (nmol/mg protein)	$\Delta\psi$ (mV)[a]	
	Malate/glutamate	Succinate
0	−211 ± 2.1	−213 ± 2.6
400	−205 ± 3.6	−206 ± 3.6*
600	−202 ± 3.7*	−203 ± 2.3*
800	−196 ± 4.3*	−197 ± 3.9*
1000	−193 ± 1.5**	−195 ± 4.5*

[a] Values are means ± standard error of the mean of three different mitochondrial preparations. *$p < 0.05$; **$p < 0.01$ vs. control (DMSO).

Due to metolachlor low solubility in water and its high partition coefficient in octanol/water (Miller et al., 1997; Weber et al., 2006), a significant incorporation of this herbicide into biological membranes is predictable. Microorganisms, including the strain of *B. stearothermophilus* used in this work, have revealed themselves as suitable models to study membrane-mediated drug toxicity, reflected in growth inhibition and perturbations of membrane-dependent cell functions (Jurado et al., 1987; Silver, 1996; Donato et al., 1997; Rosa et al. 2000; Nielsen and Winding, 2002; Luxo et al., 2003). Our results show that metolachlor inhibits growth of *B. stearothermophilus* at concentrations ranging from 100 to 600 μM (Table 1). However, the effective concentrations to reduce growth parameters by 50% (in the 400–500 μM range) are significantly higher than those for other pesticides, e.g. the organochlorine DDT (Donato et al., 1997) or the organotin TBT (Martins et al., 2005). Consistently, metolachlor's EC50 for oxygen consumption of bacterial protoplasts elicited by NADH (1.2 μmol/mg of protein, Table 2) is also much higher (two to three order of magnitude) than those found for DDT (Donato et al., 1997) and TBT (Martins et al., 2005). When a mixture of ascorbate and TMPD was used as respiratory substrate, the oxygen consumption rate was not sensitive to the presence of metolachlor in the range of 0.5–5.0 μmol/mg of protein. Therefore, metolachlor impairs the respiratory activity of *B. stearothermophilus* exerting its action upstream the cytochrome c oxidase segment. This finding concurs with studies with other pesticides whose effects on the activities of respiratory complexes were evaluated in membranes isolated from bacterial cells (Donato et al., 1997; Monteiro et al., 2005). However, further clarification of the effects of pesticides on the respiratory system of *B. stearothermophilus* is technically difficult, since inhibitors for the different segments of the respiratory system of this bacterium are not known, excepting CN^- (an inhibitor of the cytochrome c oxidase). Thus, we carried out additional studies using rat liver mitochondria, as a suitable model to assess bioenergetics-mediated toxicity of xenobiotics.

The results presented in this study show that metolachlor (up to 1.0 μmol/mg of protein) interferes with mitochondrial bioenergetics, inhibiting state 3 respiration (Fig. 3A) and FCCP-uncoupled respiration (data not shown), energized by either malate/glutamate or succinate. In agreement with these results, RCR index and ADP/O ratio were depressed (Fig. 3B). These results demonstrate that both Complexes I and II of the mitochondrial respiratory chain are sensitive to metolachlor. Accordingly, metolachlor-induced $\Delta\psi$ dissipation and depression of the phosphorylation rates (Fig. 3C and Table 3) can be explained by the inhibitory action of the herbicide exerted in Complexs I and II of the respiratory system. This assumption is supported by the parallel depressive effect on state 3- and FCCP-respiration dependent on both malate/glutamate and succinate (Fig. 3A) and the absence of effects when a mixture of ascorbate + TMPD was used as substrate (data not shown). The low stimulatory effect of state 4 respiration (Fig. 3A), suggesting a low ability of the compound to induce inner membrane permeabilization, excludes a significant involvement of cations gradient on $\Delta\psi$ dissipation and on depression of the phosphorylation rates promoted by metolachlor. Therefore, the toxicological effects of metolachlor on rat liver mitochondrial bioenergetics, expressed by its inhibitory action on mitochondrial respiratory chain for Complexes I and II, may be relevant to understand the mechanism responsible for its toxic action. However, it is needed to emphasise that metolachlor effects on mitochondria bioenergetics were detected at concentrations notably higher than other pesticides (Moreno and Madeira, 1991), as above referred for *B. stearothermophilus*, and in the same concentration range of carbaryl (Moreno et al., 2007), an insecticide of the group of carbamates that is generally considered a relatively safe compound.

It was also demonstrated that metolachlor affected the respiratory activity of *B. stearothermophilus* and the bioenergetics of rat liver mitochondria at the same concentration range (0.4–5 μmol/mg of protein), indicating a similar sensibility of these models for chemical toxicity.

In conclusion, by using *B. stearothermophilus* and rat liver mitochondria as *in vitro* biological models, our studies showed that metolachlor is less toxic than other pesticides, at cellular and subcellular levels. This conclusion is in quite agreement with available toxicological data (Stevens et al., 1991; Kontchou and Gschwind, 1998; Extoxnet) showing that this herbicide is slightly toxic for humans and non-toxic or moderately toxic for other organisms. The good parallelism observed between metolachlor data provided by these two experimental models and those reported in the literature are in accordance with the results of previous studies performed with other compounds (Donato et al., 1997; Monteiro et al., 2003, 2008). Taking into account these achievements, we suggest that these biological systems are good tools for chemical toxicity screening, reducing costly animal experimentation and alleviating associated ethics issues.

Acknowledgments

This study was supported by Portuguese Research Council (FCT), Portugal, Environment and Life Science Institute (IAV) of the University of Coimbra, Institute of Marine Research (IMAR) and Center for Neuroscience and Cell Biology (CNC).

References

Accinelli, C., Hashim, M., Epifani, R., Schneider, R.J., Vicari, A., 2006. Effects of the antimicrobial agent sulfamethazine on metolachlor persistence and sorption in soil. Chemosphere 63, 1539–1545.

Aguilar, C., Ferrer, I., Borrull, F., Marcé, R.M., Barceló, D., 1998. Monitoring of pesticides in river water based on samples previously stored in polymeric cartridges followed by on-line solid-phase extraction-liquid chromatography–diode array detection and confirmation by atmospheric pressure chemical ionization mass spectrometry. Analytica Chimica Acta 386, 237–248.

Albanis, T.A., Hela, D.G., Sakellarides, T.M., Konstantinou, I.K., 1998. Monitoring of pesicide residues and their metabolites in surface and underground waters of Imathia (N.Greece) by means of solid-phase extraction disks and gas chromatography. Journal of Chromatography A 823, 59–71.

Bragadin, M., 2006. The mitochondria: ATP synthesis and its inhibition by toxic compounds. In: Moreno, A.J.M. (Ed.), Mitochodrial Pharmacology and Toxicology. Transworld Research Network, Kerala, India.

Cardoso, S., Santos, R.X., Carvalho, C., Correia, S., Pereira, G.C., Pereira, S.S., Oliveira, P.J., Santos, M.S., Proença, T., Moreira, P.I., 2008. Doxorubicin increases the susceptibility of brain mitochondria to Ca^{2+}-induced permeability transition and oxidative damage. Free Radical Biology and Medicine 45, 1395–1402.

Cerejeira, M.J., Viana, P., Batista, S., Pereira, T., Silva, E., Valerio, M.J., Silva, A., Ferreira, M., Silva-Fernandes, A.M., 2003. Pesticides in Portuguese surface and ground waters. Water Research 37, 1055–1063.

Chance, B., Williams, G.R., 1956. The respiratory chain and oxidative phosphorylation. Advance Enzymology 17, 65–134.

Donato, M.M., Jurado, A.S., Antunes-Madeira, M.C., Madeira, V.M.C., 1997. Comparative study of the toxic actions of DDT and DDE on the growth and respiratory activity of a microorganism used as a model. Applied and Environmental Microbiology 63, 4948–4951.

Duchen, M.R., 2004. Roles of mitochondria in health and disease. Diabetes 53, 96–102.

Eisenbrand, G., Pool-Zobel, B., Baker, V., Balls, M., Blaauboer, B.J., Boobis, A., Carere, A., Kevekordes, S., Lhuguenot, J.C., Pieters, R., Kleiner, J., 2002. Methods of in vitro toxicology. Food and Chemical Toxicology 40, 193–236.

Estabrook, R.W., 1967. Mitochondrial respiratory control and the polarographic measurements of ADP/O ratios. Methods Enzymology 10, 41–47.

Extoxnet, <http://extoxnet.orst.edu/pips/metolach.htm>.

Fernandes, M.A.S., Pereira, S.P.S., Jurado, A.S., Custódio, J.B.A., Santos, M.S., Moreno, J.M., Duburs, G., Vicente, J.A.F., 2008. Comparative effects of three 1,4-dihydropyridine derivatives [OSI-1210, OSI-1211 (etaftoron), and OSI-3802] on rat liver mitochondrial bioenergetics and on the physical properties of membrane lipid bilayers: Relevance to the length of the alkoxyl chain in positions 3 and 5 of the DHP ring. Chemico-Biological Interactions 173, 195–204.

Gazotti, P., Malmstron, K., Crompton, M.A., 1979. Laboratory manual on transport and bioenergetics. In: Carafoli, E., Semenza, G. (Eds.), Membrane Biochemistry. Springer, New York, pp. 62–69.

Gornall, G., Bardawill, C.J., David, M.M., 1949. Determination of serum proteins by means of the biuret reaction. Journal of Biological Chemistry 177, 751–766.

Huber, A., Bach, M., Frede, H.G., 2000. Pollution of surface waters with pesticides in Germany: modeling non-point source inputs. Agriculture Ecosystems and Environment 80, 191–204.

Jurado, A.S., Santana, A.C., Costa, M.S., Madeira, V.M.C., 1987. Influence of divalent cations on the growth and morphology of *Bacillus stearothermophilus*. Journal of General Microbiology 133, 507–513.

Kamo, N., Muratsugu, M., Hongoh, R., Kobatake, N., 1979. Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl-phosphonium and relationship between proton electrochemical potential and phosphorylation potential in stade state. Journal of Membrane Biology 49, 105–121.

Koeman, J.H., 1984. Review of environmental problems caused by the use of fertilizers and pesticides. In: Winteringham, F.P.W. (Ed.), Environment and Chemicals in Agriculture/Proceedings of a Symposium hold in Dublin. Elsevier Applied Science Publishers Ltd., Amsterdam, pp. 19–27.

Kontchou, C.Y., Gschwind, N., 1998. Interactions and biodegradation of the herbicide metolachlor with different surfaces. Ecotoxicology and Environmental Safety 40, 29–33.

Luxo, C., Jurado, A.S., Madeira, V.M.C., Silva, M.T., 2003. Tamoxifen induces ultrastructural alterations in membrane of *Bacillus stearothermophilus*. Toxicology in Vitro 17, 623–628.

Martins, J.D., Jurado, A.S., Moreno, A.J.M., Madeira, V.M.C., 2005. Comparative study of tributyltin toxicity on two bacteria of the genus *Bacillus*. Toxicology in Vitro 19, 943–949.

Martins, J.D., Monteiro, J.P., Antunes-Madeira, M.C., Jurado, A.S., Madeira, V.M.C., 2003. Use of the microorganism *Bacillus stearothermophilus* as a model to evaluate toxicity of the lipophilic environmental pollutant endosulfan. Toxicology in Vitro 17, 595–601.

Miller, J.L., Wollum, A.G., Weber, J.B., 1997. Degradation of carbon-14-atrazine and carbon-14-metolachlor in soil from four depths. Journal of Environmental Quality 26, 633–638.

Monteiro, J.P., Jurado, A.S., Moreno, A.J.M., Madeira, V.M.C., 2005. Toxicity of methoprene as assessed by the use of a model microorganism. Toxicology in Vitro 19, 951–956.

Monteiro, J.P., Martins, J.D., Luxo, P.C., Jurado, A.S., Madeira, V.M.C., 2003. Molecular mechanisms of the metabolite 4-hydroxytamoxifen of the anticancer drug tamoxifen, use of a model microorganism. Toxicology in Vitro 17, 629–634.

Monteiro, J.P., Oliveira, P.J., Moreno, A.J.M., Jurado, A.S., 2008. Disruption of hepatic mitochondrial bioenergetics is not a primary mechanism for the toxicity of methoprene – relevance for toxicological assessment. Chemosphere 72, 1347–1354.

Moreno, A.J., Madeira, V.M., 1991. Mitochondrial bioenergetics as affected by DDT. Biochimica Biophysica Acta 1060, 166–174.

Moreno, A.J.M., Serafim, T.L., Oliveira, P.J., Madeira, V.M.C., 2007. Inhibition of mitochondrial bioenergetics by carbaryl is only evident for higher concentrations – relevance for carbaryl toxicity mechanisms. Chemosphere 66, 404–411.

Nielsen, M.N., Winding, A., 2002. Microorganisms as indicators of soil health. National Environmental Research Institute, Denmark. Technical Report No. 388.

Pereira, C.V., Moreira, A.C., Pereira, S.S., Machado, N.G., Carvalho, F.S., Sardão, V.A., Oliveira, P.J., 2009. Investigating drug-induced mitochondrial toxicity: a biosensor to increase drug safety? Current Drug Safety 4, 34–54.

Rosa, S.M.L.J., Antunes-Madeira, M.C., Matos, M.J., Jurado, A.S., Madeira, V.M.C., 2000. Lipid composition and dynamics of cell membranes of *Bacillus stearothermophilus* adapted to amiodarone. Biochimica Biophysica Acta 1487, 286–295.

Scatena, R., Bottoni, P., Botta, G., Martorana, G.E., Giardina, B., 2007. The role of mitochondria in pharmacotoxicology: a reevaluation of an old, newly emerging topic. American Journal Physiology and Cell Physiology 239, 12–21.

Silver, S., 1996. Bacterial resistances to toxic metal ions – a review. Gene 179, 9–19.

Starkov, A.A., Wallace, K.B., 2002. Structural determinants of fluorochemical-induced mitochondrial dysfunction. Toxicological Science 66, 244–252.

Stevens, J.T., Summer, D.D., 1991. In: Hayes, W.J., Laws, E.R. (Eds.), Handbook of Pesticide Toxicology. Academic Press, New York, p. 11.

Weber, J.B., Warren, R.L., Swain, L.R., Yelverton, F.H., 2006. Physicochemical property effects of three herbicides and three soils on herbicide mobility in field lysimeters. Crop Protection 26, 299–311.

Wisdom, C., Welker, N.E., 1973. Membranes of *Bacillus stearothermophilus*: factors affecting protoplast stability and thermstability of alkaline phosphatase and reduced nicotinamide adenine dinucleotide oxidase. Journal of Bacteriology 114, 1336–1345.

Please cite this article in press as: Pereira, S.P., et al. Toxicity assessment of the herbicide metolachlor comparative effects on bacterial and mitochondrial model systems. Toxicol. In Vitro (2009), doi:10.1016/j.tiv.2009.06.032