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Potential of the microbial assay for risk assessment (MARA) for assessing ecotoxicological effects of herbicides to non-target organisms

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Abstract Many microbiotests that have been proposed for use in the risk assessment of environmental pollutants have the drawback of lacking relevant published data on various aspects of their test application possibilities and therefore do not receive the regulatory recognition which they may deserve. The MARA bioassay lacks published data for many relevant environmental pollutants, particularly pesticides and this may limit its use in regulatory framework. The present study has assessed the sensitivity of the MARA bioassay relative to other established bioassays (*Daphnia magna* and *Oreochromis niloticus*) to two widely used herbicide formulations: Roundup (having glyphosate as active ingredient) and Herbextra (with the active ingredient being 2,4-dichlorophenoxyacetic acid—2,4-D). Roundup was found to be more toxic than Herbextra in all three bioassays. The *D. magna* EC50 s obtained for Roundup and Herbextra were respectively 5.55 and 356.61 mg/l while the LC50 s for *O. niloticus* were 11.30 and 222,28 mg/l respectively. In the case of the MARA bioassay microbial toxic concentrations (MTCs) for individual species ranged from 6.85 to 468 mg/l with an overall mean MTC of 101.82 mg/l for glyphosate and from 74.67 to 13,333 mg/l for 2,4-D giving an overall mean MTC of 2855.88 mg/l. Although the overall MTCs from the MARA bioassay were much higher than the LC50 s and EC50 s from the fish and daphnia bioassays respectively, the most sensitive MARA organism for each of the herbicides had MTCs that were comparable to or lower than the corresponding endpoints from the other bioassays implying that the MARA assay is a potentially useful bioassay for risk assessment of pesticides.

Keywords MARA · Glyphosate · Microbial assays · 2 · 4-D · Herbicides

Introduction

Pesticides play a key role in enabling agricultural intensification by protecting crops from damage by insect pests and pathogenic diseases, and by reducing competition from weed plants. However, because pesticides are designed to be biologically active, they may also be hazardous to certain non-target organisms. They are also typically introduced into the agro-ecosystem in large quantities (Carriquiriborde et al. 2014) with herbicide usage more than doubled in the last three decades (Mason et al. 2003). Consequently, it is necessary to assess the potential risks of pesticides to non-target organisms.

A range of acute toxicity bioassays have been developed to establish the ecotoxicological impact of contaminants on aquatic organisms (Farre and Barcelo 2003). However, many of the standard bioassays are still laborious, time-consuming and require large sample volumes (Parvez et al. 2006). Biotests for toxicity detection in effect-directed analysis (EDA) need to have low volume requirement. In addition, they need to be rapid, sensitive, and reproducible, have high throughput and the power to discriminate toxic and non-toxic samples. In the past two decades there have been intensive efforts to scale down standard toxicity tests in an effort to facilitate risk assessment and environmental monitoring amongst other uses (Blaise 1998; Blaise and Férard 2005; Blaise et al. 1998; Nalewajko and Olaveson 1998; Wells 1999; Wells et al. 2001). In addition, large

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numbers of alternative microscale toxicity tests have been proposed to either replace or complement standard tests (Fai and Grant 2009; Fai et al. 2007; van Beelen 2003; Wadhia 2008) and have several advantages such as: they are highly reproducible, rapid, easy to handle, do not consume too much sample, and may be advanced to a real high-throughput instrument by using microtiter plates (Brack 2003). Several of these tests have also been developed into test kits (Janssen et al. 2000). Microplate-based bioassays also called microbiotests (Blaise 1998; Cerejeira et al. 1998; Fochtman et al. 2000; Persoone et al. 2003; Wadhia and Thompson 2007) meet most of these conditions in that they are easy to manipulate, cost-effective, space-saving, provide a large number of replicates, have the potential for automation and reduce contamination (Blaise 1998; Blaise et al. 1998; Caux et al. 1992). Microbiotests using microbial species have additional advantages including substantially reduced duration of the tests owing to short generation times, absence of ethical issues usually associated with vertebrate species (Wadhia and Thompson 2009b). Acute bioluminescence inhibition of the bacteria *Vibrio fischeri* has become the predominant microbiotest having all above mentioned advantages. However, it has drawbacks for hazard assessment such as its inability to detect many ecotoxicologically relevant specific effects (e.g. of herbicides, insecticides, and antibiotics) and the fact that long-term effects are hardly detectable in this test system (Brack 2003). On the other hand the microbial assay for risk assessment (MARA) is an innovative ''battery of tests within a test'' with significant potential (Gabrielson et al. 2003; Wadhia and Thompson 2007). It is a 24 h multi-species test consisting of 11 taxonomically diverse microbial strains used to assess the ecotoxicity of chemicals and environmental samples. The strains show different sensitivities to different chemicals and the resulting array of 11 inhibition values gives a toxic fingerprint of the chemicals tested (Gabrielson et al. 2003; Wadhia et al. 2007; Wadhia and Thompson 2007; Wadhia and Thompson 2009a). The growth inhibition of these microorganisms due to toxic chemicals and environmental samples is measured in parallel. This test has undergone rigourous intra and inter-laboratory testing which established its reproducibility and repeatability (Wadhia 2008; Wadhia et al. 2007). There is however, limited published data on MARA toxicity values for pesticides and many other important environmental pollutants and this may limit its use in regulatory framework. It is therefore very important to explore all aspects of its applicability in ecotoxicological testing and risk assessment. The aims of this study were to assess ecotoxicity of two widely used herbicide formulations: Roundup (glyphosate as active ingredient) and Herbextra (2.4-dichlorophenoxycetic acid—2,4-D) using MARA test and comparison of the results with the sensitivity of other toxicity tests, namely *Daphnia magna* and *Oreochromis niloticus* acute tests.

Materials and methods

Test materials, chemicals and organisms

All materials and equipment for the MARA test were obtained from the UK National Collection of Industrial, Food and Marine Bacteria (NCIMB, Bucksburn, Aberdeen) and consisted of MARA plates (containing lyophilised MARA microorganisms), microbial growth medium (Phytone peptone), Hp scanner7400c, dye (1 % terazolium red solution) and MARA software. Two herbicide formulations were used in the present study: herbextra 720 SL (Ader CAM, 720 g/l of 2,4-D) and Roundup (Monsanto®, 360 g/l glyphosate). Fingerlings of *O. niloticus* of average size ± SD 3.97 ± 0.66 cm and average weight ± SD of 1.22 ± 0.54 g were obtained from a local fish farmer. *D. magna* ephippia were obtained from MicroBioTests Inc Belgium and hatched in our laboratory into neonates which were used for the bioassays.

MARA test

The MARA test was performed in triplicate according to the standard protocol described by Wadhia et al. (Wadhia et al. 2007). In this work, the microbial species used consisted of ten bacterial species: *Microbacterium sp*., *Brevundimonas diminuta, Citrobacter freundii, Comamonas testosterone, Enterococcus casseliflavus, Delftia acidovorans, Kurthia gibsonii, Staphylococcus warnerii, Pseudomonas aurantica, Serratia rubidaea,* and one yeast species, *Pichia anomalia.* (Gabrielson et al. 2003; Wadhia and Thompson 2007). A measure of the growth of the organisms over a range of concentrations of the test substances was determined with the reduction of tetrazolium red (TTC). Three fold serial dilution series were carried out in both cases to obtain the following concentration ranges: 177.78, 533.33, 1600.00, 4800.00, 14,400.00 and 43200.00 mg 2,4-D/l in the case of herbextra and 2.96, 8.89, 26.67, 80.00, 240.00 and 720.00 mg glyphosate/l for Roundup. A flatbed scanner was utilised to capture an image of the test plate and the scan was subsequently analysed using a purpose-built software.

Fish and daphnid toxicity tests

The acute toxicity tests with *O. niloticus* were carried out according to standard OECD protocol (OECD 1992) in which the fingerlings of this fish species were exposed to a range of concentrations of each pesticide separately in a static system for 96 h. The concentrations used for

herbextra were: 22.50, 45.00, 90.00, 180.00 and 360.00 mg 2,4-D/l while for Roundup the concentrations used were 0.63, 1.25, 2.50, 5.00, 10.00, 20.00, and 40.00 mg glyphosate/l. Mortality was recorded every 24 h and data used to establish dose–response curves and calculate LC50 s.

The *D. magna* acute immobilisation test was carried out according to standard OECD protocol (OECD 2004). The herbextra concentrations used were: 11.25, 22.5, 45.00, 90.00, 180.00, 360.00 and 720.00 mg 2,4-D/l while the following Roundup concentrations were used: 0.49, 1.48, 4.44, 13.33 and 40.00 mg glyphosate/l. Neonate immobilization was recorded at 24 and 48 h and data obtained was used to establish dose–response curves and calculate EC50 s.

Statistical analysis

Statistical analysis of results from the MARA bioassay was carried out using the MARA software, Microsoft Excel and SPSS 12.0.1 for Windows with graphs plotted in Sigma Plot 2000. The MARA plate images obtained from the scanner were saved in Microsoft Excel files. For analysis theses images were copied from Excel files and loaded one at a time into the MARA software. The MARA software assigned numerical values to each well in the plate, which represented the amount of microbial growth. The software also calculated the percentage growth and growth inhibition of micro-organisms in each well relative to the respective controls as well as microbial toxic concentration (MTC) values for each organism and an overall mean MTC for the 11 MARA organisms in each plate (Gabrielson et al. 2003; Wadhia et al. 2007). Mean MTCs were therefore calculated for each MARA species from the three replicates for each of the herbicides tested and an overall MTC for each herbicide from the three replicates obtained. The MTC is calculated by the MARA software according to the formula $MTC = c_{\min} d^{(P_{tot}/P_0)-0.5}$ developed by Gabrielson et al. (2003), where c_{min} = lowest concentration in the gradient, P_0 - = pellet size in the control well, d = dilution factor and P_{tot} = the sum of the pellet sizes in all wells exposed to the concentration gradient of the chemical to be tested.

The concentrations corresponding respectively to 50 % *O. niloticus* mortality (LC50 s) and *D. magna* immobilisation (EC50 s) for each herbicide were also calculated at various exposure times using the Microsoft Excel macro, REGTOX (Vindimian 2009), which models the dose–response relationship with the non-linear Hill equation.

Results

Responses of MARA species to glyphosate and 2,4-D

The dose–response curves of MARA species exposed to glyphosate (Fig. 1a) and 2,4-D (Fig. 1b) show the diverse responses typical of MARA species to each of the herbicides. MTCs obtained for each MARA species (Fig. 2) also reflect the same diversity in responses. MTCs for glyphosate ranged from 6.85 to 468 mg/l with a mean value of 101.82 mg/l while MTCs for 2,4-D ranged from 74.67 to 13,333 mg/l with a mean MTC of 2855.88 mg/l. Glyphosate was more toxic than 2,4-D to all MARA species although the magnitude of the difference was not the same for the different MARA species (Fig. 2). The mean MTCs for both herbicides also reflected this trend with the mean MTC for 2,4-D being 28 times higher than that for glyphosate. *K. gibsonii* was the most sensitive MARA species to glyphosate followed by *S. warnerii* and then *B. diminuta*, *E. casseliflavus, P. anomalia* and *D. acidovorans* in decreasing sensitivity order. The most tolerant species to glyphosate was *P. aurantica.* In the case of 2,4-D the most sensitive MARA species was *D. acidovorans* followed by *Microbacterium* sp. and then *K. gibsonii*, *E. casseliflavus* and *S. warnerii* with the most tolerant species to 2,4-D being *P. aurantica* (Fig. 2).

Responses of *Oreochromis niloticus* to glyphosate and 2,4-D

It can be seen from Fig. 3a that at 24 h there was very little mortality ($\leq$ 20 %) of *O. niloticus* fingerlings up to a concentration of 10 mg/l glyphosate after which there was a sharp increase in mortality to 100 % at 20 mg/l. This gave a steep dose–response curve and a 24 h LC50 of 11.59 mg/l (Table 1). From 48 to 96 h there was little additional mortality over time and consequently no major change in the LC50 s. A 96 h LC50 of 11.30 mg/l was obtained for glyphosate (Table 1).

In the case of 2,4-D no death occurred in concentrations up to 90 mg/l throughout the entire duration of the test. Partial mortality was only observed in one of the five test concentrations (180 mg/l) at 24 h with no further mortality occurring for the rest of the test (Fig. 2b). This gave an LC50 of 222.28 mg/l (Table 1). Glyphosate was therefore more toxic than 2,4-D to *O. niloticus* with the 2,4-D LC50 being 19 times higher than that for glyphosate.

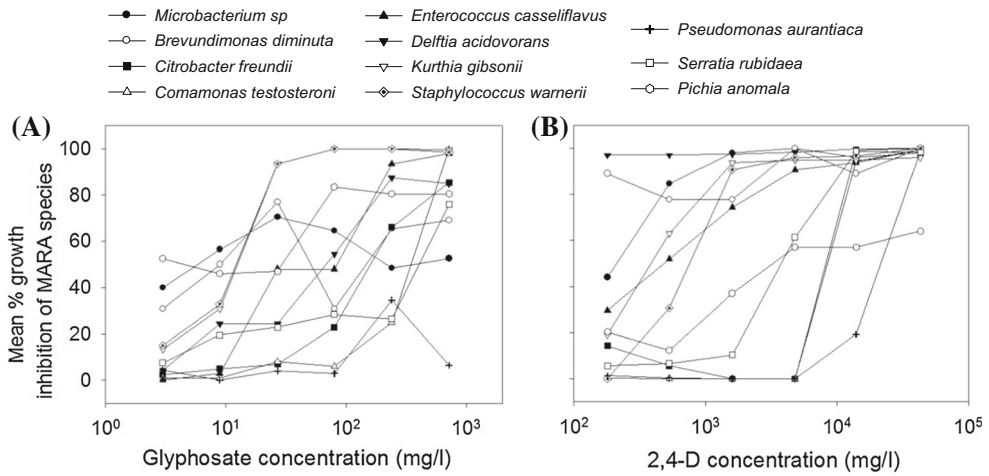

Fig. 1 MARA species responses to herbicides. **a** Glyphosate, **b** 2,4-D

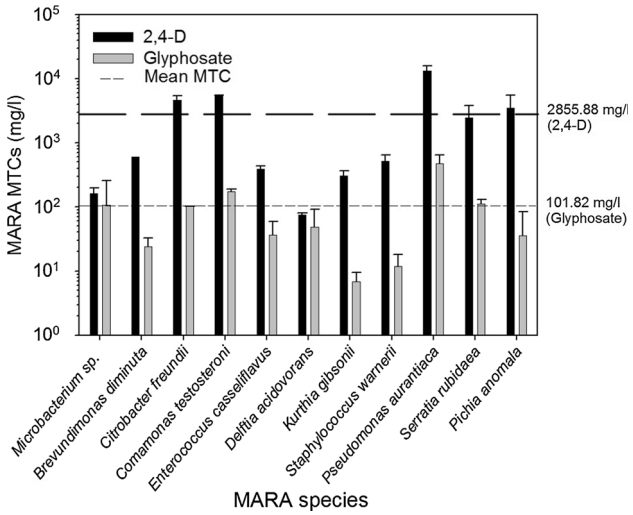

Fig. 2 MARA species MTCs for Glyphosate and 2,4-D

Responses of *Daphnia magna* to glyphosate and 2,4-D

There was a clear dose–response relationship observed after 24 h of exposure to glyphosate with 100 % immobilisation occurring at 40 mg/l glyphosate (Fig. 4a) giving an EC50 of 16.61 mg/l glyphosate (Table 1). The response increased over time and by 48 h the EC50 decreased to 5.55 mg/l (Table 1).

A reliable EC50 could not be obtained after 24 h of exposure to 2,4-D as no immobilisation was observed up to concentrations of 360 mg/l and only the highest concentration of 720 mg/l led to immobilisation. However, by 48 h partial immobilisation responses were obtained at various exposure concentrations with 100 % immobilisation occurring at the highest concentration (Fig. 4b) giving an EC50 of 356.61 mg/l (Table 1). Once more glyphosate was found to be more toxic than 2,4-D to *D. magna* with the 2,4-D EC50 being 64 times higher.

Comparing sensitivities of MARA, *Oreochromis niloticus* and *Daphnia magna* sensitivity to both herbicides

For comparison purposes, the MARA MTCs were compared with the 96 h LC50 from the *O. niloticus* and the 48 h EC50 from the *D. magna* bioassays. These values are

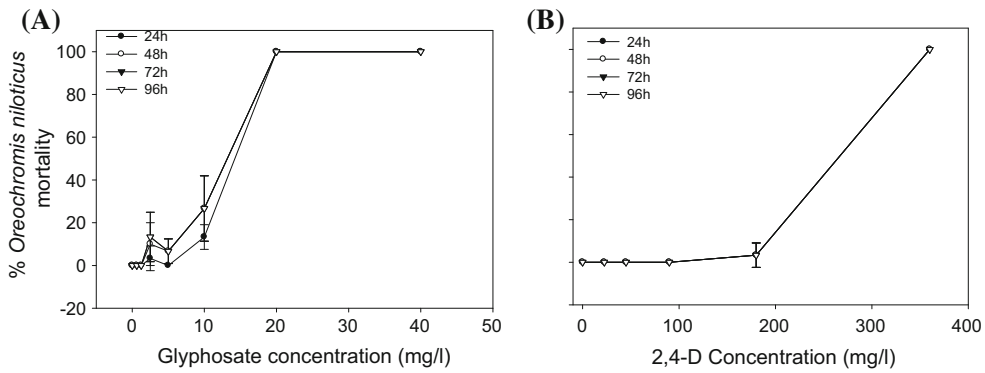

Fig. 3 *Oreochromis niloticus* responses to herbicides. **a** Glyphosate, **b** 2,4-D

Table 1 Calculated LC50 s and EC50 s at various exposure times respectively for *Oreochromis niloticus* and *Daphnia magna* exposed to Roundup (Glyphosate) and Herbextra (2,4-D)

Exposure period (h)	*Oreochromis niloticus* LC_{50} (confidence interval) (mg/l)		*Daphnia magna* EC_{50} (confidence interval) (mg/l)	
	Glyphosate	2,4-D	Glyphosate	2,4-D
24	11.59 (11.25–11.71)	222.28 (209.86–228.11)	16.61 (12.12–16.42)	Not attained
48	11.30 (10.33–11.56)	222.28 (209.86–228.11)	5.55 (4.01–6.41)	356.61 (268.18–368.10)
72	11.30 (10.26–11.60)	222.28 (209.86–228.11)	–	–
96	11.30 (10.17–11.57)	222.28 (209.86–228.11)	–	–

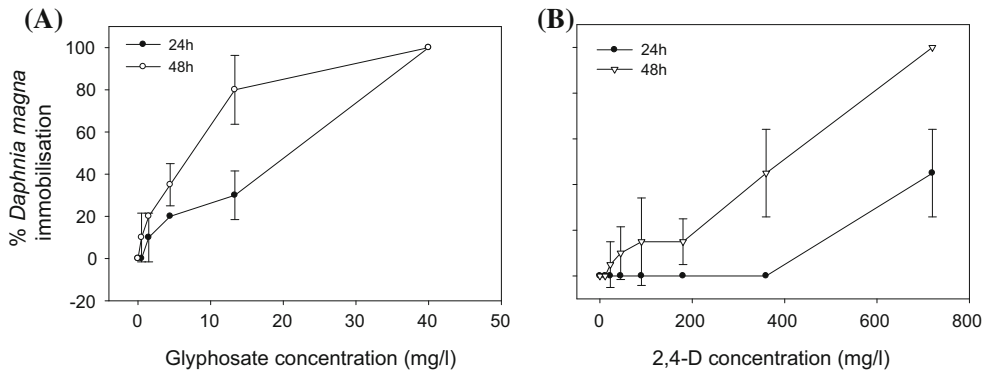

Fig. 4 *Daphnia magna* responses to herbicides. **a** Glyphosate, **b** 2,4-D

referred to as the toxicity endpoints for these bioassays in the text that follows.

Glyphosate was found to be more toxic in all three bioassays, but the magnitude of difference in toxicity of the two herbicides was distinct in each bioassay. The overall mean MARA MTC for 2,4-D was 28 times higher than that of glyphosate while the *O. niloticus* 96 h LC50 for 2,4-D was 19 times higher and the *D. magna* 48 h EC50 for 2,4-D was 64 times higher than the corresponding values for glyphosate.

The overall mean MARA MTCs for both herbicides were higher than either the 96 h LC50 or 48 h EC50 obtained respectively from the fish and daphnia bioassays. In the case of glyphosate the overall mean MARA MTC was respectively 9 and 18 times higher than the corresponding toxicity endpoints for the fish and daphnia bioassays. Looking at 2,4-D the overall mean MARA MTC was still higher than toxicity endpoints for both assays but this time there was a higher difference with the fish bioassay as the MARA MTC was 12.8 times higher than the fish bioassay endpoint but only 8 times higher than the daphnia bioassay endpoint. However, the most sensitive MARA species to glyphosate (*K. gibsonii*) had a mean MTC of 6.85 mg/l which is lower (and therefore the species was more sensitive) than the *O. niloticus* endpoint for glyphosate (Table 1) although slightly higher than the *Daphnia magna* bioassay endpoint of 5.55 mg/l. On the other hand, the most sensitive MARA species to 2,4-D (*D. acidovorans*) had a mean MTC of 74.7 mg/l which is much lower than the endpoints from both the *O. niloticus* and *D. magna* bioassays (Table 1).

Discussion

Glyphosate [*N*-(phosphonomethyl) glycine] is a broad spectrum, post emergent herbicide and is among the most widely used agricultural chemicals globally (Annett et al. 2014; Duke and Powles 2008; Kolpin et al. 2006; Tsui and Chu 2003). The creation of glyphosate tolerant crop species has significantly increased the demand and use of glyphosate-based herbicides (e.g. Roundup) and has also increased the risk of exposure to non-target species (Annett et al. 2014; Duke and Powles 2008) but there is a paucity of data on the toxicity of the formulated products (Tsui and Chu 2003). 2,4-D is another widely used herbicide (Sarikaya and Selvi 2005) which has the potential to affect non-target organisms. Pesticides pollution therefore still presents a major problem in the world and risk assessment of pesticides is a major concern for many governments. Scientifically sound risk assessment and management of chemicals is the basis for any chemical control and risk reduction measures and ultimately provides a basis for the sustainable use of substances (Backhaus et al. 2010). Effect

assessments by determining safe concentrations based on laboratory toxicity data of the compound in question is an important component of pesticides risk assessment (Ansara-Ross et al. 2008). The present study has examined the potential of the MARA bioassay to assess the toxicity of two herbicides—glyphosate and 2,4-D by comparing the sensitivity of the MARA bioassay to two bioassays using a local fish species (*O. niloticus*) and the standard test organism, *D. magna*.

Although the toxicity of glyphosate-based herbicides is known to be highly variable, being affected by the type of surfactant used in the formulation as well as environmental factors like pH and suspended soil particles (e.g. Annett et al. 2014; Tsui and Chu 2003), *D. magna* EC50 s and *O. niloticus* LC50 s obtained in the present study for glyphosate are in agreement with published values for these organisms under similar test conditions (Cuhra et al. 2013; Folmar et al. 1979; Jiraungkoorskul et al. 2002). In the present study there was no change in fish mortality after 48 h of exposure to glyphosate or after 24 h exposure to 2,4-D, leading to very little change in the respective LC50 s values reported. This is similar to the results of Jiraungkoorskul et al. (2002) and can be explained by the fact that these herbicides have very short half lives and the tests carried out were acute static non-renewal bioassays (Duke and Powles 2008). Because of this low persistence, repeated applications of this herbicides are practiced for the control of weeds in agricultural fields and thereby, large quantities find their ways into the water bodies (Ayoola 2008). Glyphosate mode of action is unique in that it is the only molecule that is highly effective at inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase of the shikimate pathway which links metabolism of carbohydrates to biosynthesis of aromatic compounds. This pathway exists in higher plants and microorganisms but not in animals (Duke and Powles 2008; Herrmann and Weaver 1999). However, the MARA species were not appreciably more sensitive to glyphosate than either *D. magna* or *O. niloticus* in the present study probably because the sole target for glyphosate is the penultimate enzyme of the shikimate pathway in plants while the shikimate pathway in microorganisms is regulated by feedback inhibition and by repression of the first enzyme (Herrmann and Weaver 1999).

In all cases in the present study glyphosate was more toxic than 2,4-D. 2,4-D is an analogue compound to the plant hormone indole-3-acetic acid. Its hormone-like character and general structural similarity to that of indole-3-acetic acid suggests that it may act indirectly by altering the activity of this natural plant hormone (Goldacre 1949). This hormone is neither found in animals nor in microorganisms, which explains the low toxicity of 2,4-D to these organisms. In the case of glyphosate, although the target pathway for glyphosate is not found in animals, it has been shown that fish and aquatic vertebrates are more sensitive to glyphosate than other animals (Duke and Powles 2008). Roundup has been shown to inhibit acetylcholinesterase enzyme as well as interfere with antioxidant defenses in fish (Modesto and Martinez 2010).

The sensitivities of the different MARA species varied widely for each of the two herbicides in the present study. This is typical of the MARA bioassay and previous studies have attributed this to the wide genetic diversity of the selected MARA species (Fai and Grant 2010; Gabrielson et al. 2003). In addition, considerable differences in sensitivity among micro-organisms is known to exist (DeLorenzo et al. 2001). Although the most tolerant MARA species (*P. aurantica*) happened to be the same for both herbicides in the present study, the most sensitive MARA species was different for the two herbicides. This is a very important feature and a great advantage of the MARA bioassay as it increases the likelihood of detecting toxic effects from a wide range of contaminants (Gabrielson et al. 2003). The overall mean MARA MTCs for both herbicides in the present study were much higher than the corresponding LC50 s and EC50 s in the fish and daphnia bioassays respectively. However, the MTCs for the most sensitive MARA species for each of the herbicides were either comparable to or lower than the corresponding LC50 s and EC50 s from the fish and daphnia bioassays respectively. This result agrees with published works which show the sensitivity of the MARA assay for a number of chemicals to compare favourably with other assays including the *D. magna* bioassays (Fai and Grant 2010; Gabrielson et al. 2003). It is also interesting to note that the glyphosate MTC for the most sensitive MARA species in the present study is lower than published IC50 for Roundup® using the Microtox® bioassay (Tsui and Chu 2003), a very widely used microbial assay. This once more demonstrates the advantage of the MARA bioassay being a multispecies test using eleven different microorganisms simultaneously and obtaining 11 toxicity values in each test (Wadhia and Thompson 2007) increasing the possibilities of MARA for toxicity detection.

MARA toxicity values for the herbicides glyphosate and 2,4-D have been obtained in the present study. The MARA mean MTC was not a useful toxicity endpoint due to the very large variation in the sensitivities of the various MARA species to each herbicide. The MTC of the most sensitive MARA species proved to be the most appropriate toxicity endpoint. The present study has therefore shown that the MARA bioassay is capable of assessing the effects of the herbicides glyphosate and 2,4-D to non-target organisms just as other tests like the *O. niloticus* bioassay or the standard *D. magna* acute toxicity bioassay. However, MARA has the added advantage that it is a multispecies

test with different MARA species having widely different sensitivities to each herbicide and the most sensitive MARA species being different for the two herbicides. Therefore it can be used in the place of several different tests and would be an asset in the risk assessment of pesticides.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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