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COMPARING ECOLOGICAL RISKS OF PESTICIDES ON UNICELLULAR FRESHWATER GREEN ALGA; *Pseudokirchneriella subcapitata* Using A Risk Quotient RANKING APPROACH.

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ABSTRACT

Environmental risk assessment of pesticides often uses the Risk Quotient (RQ) method to characterize risk quantitatively. Toxicological effects of pesticides (for 5 fungicides, 4 herbicides and 6 insecticides formulations) were assessed on the green alga *Pseudokirchneriella subcapitata*. For each tested pesticide, 96-h acute toxicity test was conducted and toxicity end-points and RQ values based on refinement of exposure concentrations were evaluated. Risk presumptions were presented along with the corresponding level of concern (LOC). Toxicity data indicated differential numerical ranking of pesticides within each pesticide category. For fungicides; mancozeb was found to be the most toxic and difenoconazole was the least toxic (3006 and 93 fold of toxicity; respectively). As related to herbicides, metribuzin was the most effective (1349 fold of toxicity), while cryomazine was practically non-toxic. Moreover, abamectin proved to be the extremely toxic insecticide (5511 fold of toxicity). Numerical ranking of toxicity was significantly correlated with the RQ ranking in most cases and depends on the nature of the pesticide, bioavailability and exposure concentrations. Associated risk presumptions proved that abamectin, lambda-cyhalothrin, chlorfluazuron and mancozeb pose the worst case acute risk to non-target organisms in aquatic ecosystem and consequently a regulatory action may be warranted in addition to restricted use classification (RQs=27.769; 21.568; 14.833 and 11.678; respectively exceeding LOC). Based on RQ values, risk presumption due to exposure of *P. subcapitata* to all tested insecticides represents the highly acute potential risk (RQ= 2.988- 27.769 exceeding LOC). Fungicides are also presumed to pose a high risk on aquatic ecosystem but in a trend lower than insecticides. Among tested herbicides, only metribuzin and thiobencarb are presumed to pose a high acute risk on aquatic ecosystem (RQ=2.306 and 1.167; respectively exceeding LOC). The results support the hypothesis that numerical ranking of RQs can be used for the purpose of comparing potential ecological risks and such data may add to the pesticide existing database concerning the potential adverse effects of pesticides on aquatic ecosystem.

Keywords: Pesticide toxicity, comparative risk assessment, risk ranking, green algae

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INTRODUCTION

Areas with intensive agriculture are generally highly integrated with aquatic ecosystems because of their dependence on water supply and/or drainage. These aquatic systems not only support agricultural needs but also function as an important habitat for many water organisms. With the actual application techniques of pesticides used for crop protection, however, it is inevitable that part of the sprayed amount will enter these non-target surface waters (Capri and Trevistan, 1998). As a result, bodies of water may be contaminated with a mixture of insecticides, fungicides, and herbicides. Several studies have documented that pesticide residues occur in aquatic ecosystems in agricultural areas, often with several pesticides co-occurring (Hoysaeter, 1994; Larson et al., 1999).

Aquatic ecosystems contain many species and undesirable side-effects are to be expected when these systems become contaminated. Algae are important components of the primary production in aquatic ecosystem and detrimental effects in these organisms may affect the entire food chain. *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) is a unicellular chlorophyceae algae that was widely used in studies of pollutants effects (Jonsson et al., 1998; Okamura et al., 2002) and recommended by regulatory national (CETESB, 1992; Jonsson and Maia, 1999) and international (OECD, 1984; USEPA, 1994) agencies as a test organism.

Environmental risk assessment of pesticides and other chemicals often uses the Risk Quotient (RQ) method to characterize risk quantitatively. For each ecological receptor, a RQ is a ratio of exposure to effect and calculated by dividing the estimated or actual environmental concentration by the appropriate toxicity end-point. The RQ can be used by risk analysts and decision makers to assess whether the value exceeds any predetermined threshold levels of concern (LOC) for the purpose of making comparisons of environmental risk among pesticides (Maud et al., 2001; Peterson and Hulting, 2004) and identify the need for considering a regulatory action. Consequently, the ecological relevance of estimated risk quotient is one of the main items in recent ecotoxicological research with pesticides (Leeuwangh et al., 1994; Maund et al., 1997).

The aim of this study was to assess quantitatively the potential hazards of pesticides to the freshwater organism *P. subcapitata* through measuring toxicity end-point and estimating RQs in relationship with Level of Concern (LOC).

MATERIALS AND METHODS

Chemicals: Tested pesticides were obtained from the Central Lab of pesticides; Agricultural Research Center, Ministry of Agriculture, Egypt. Their chemical names are shown in Table (1).

Test organism and culture conditions

The green alga *Pseudokirchneriella subcapitata* obtained from Faculty of Science; Mansoura University, Egypt; was used as a test organism. The stock culture was maintained according to USEPA (2002) and OECD (2002) in 250ml borosilicate Erlenmeyer flasks containing culture medium at 24 ± 2°C, under a continuous white fluorescent light of 3000–4000 lux and manually shake twice a day.

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Axenic culture was maintained in standard AAM nutrient media as described in Miller et al. (1978). Two ml of stock culture were weekly transferred into 100ml of new culture medium to maintain a continuous supply of ‘‘healthy’’ cells for the tests.

Acute toxicity test

Algal acute toxicity test was conducted using different concentrations of a pesticide in sterile algal AAM growth media. Tested concentrations of a pesticide were prepared from stock solutions on an arithmetic progression covering an expected range of toxicity from 0 to 90%. The final volume of AAM medium containing tested chemical was 50 ml. An inoculum of exponentially-growing culture of *P. subcapitata* (harvested from 4-7 days stock culture) was prepared no more than 2-3 hr prior to beginning of the test. Initial cell density for the growth inhibition test was 10,000 cells/ml. Controls containing only growth medium and algae were included. The test vessels were incubated continuously in a temperature-controlled (25°C) orbital shaker set at 100 rpm under continuous illumination provided by white fluorescent lamps. Zero-time begins when we inoculate the test flasks containing the media and the tested pesticide with the algal cells followed by incubation for 96 hr. At the end of 96 hr, the growth of the alga in terms of viable cell concentration was determined in a Neubaur hemocytometer using a phase contrast microscope (Megharaj et al., 1999) accompanied with measurement of change in pH. Growth inhibition (biomass) of the alga was used as the end point in this bioassay. All assays were conducted in duplicate. The percent inhibition values calculated relative to growth in untreated controls were used to obtain the inhibitive concentration; EC_{50} (concentration inhibitory to 50% of growth).

Estimation of risk quotient

In the deterministic approach, according to the Office of Pesticide Programs (OPP), Environmental Fate and Effects Division (EFED- EPA, 2009), a risk quotient (RQ) is a simple screening-level estimate calculated by dividing a point estimate of exposure by a point estimate of effects.

Risk quotient (RQ) = Exposure/ Toxicity

For each pesticide type (herbicide, fungicide and insecticide) and aquatic risk type, toxicities and RQs based on refinements of estimated and actual environmental exposures were ranked numerically from highest toxicity or RQ to lowest. After the risk quotient(s) was calculated, it is compared to the Agency's Level of Concern (LOC). An LOC is a policy tool that the Agency uses to interpret the risk quotient and to analyze potential risk to non-target organisms and the need to consider regulatory action.

Statistical analysis

The growth rates were calculated according to official guidelines (OECD, 2002). Percent of inhibition in algal growth (%I) relative to growth in control systems were calculated and EC_{50} (median or half maximum effective concentration) values and other statistical parameters were estimated using Probit analysis (Finney, 1971).

RESULTS

Toxicity endpoints values after exposure of *P. subcapitata* to 15 pesticides (5 fungicides, 4 herbicides and 6 insecticides) were illustrated in Table (2). The sensitivity; expressed as EC_{50}, ranged from 0.024-132.256 mg/L. The EC_{50} values of fungicides; mancozeb, copper hydroxide, copper sulfate anhydrous, probiconazole**,** flusilazole and difenoconazole were 0.044, 0.093, 0.371, 0.578, 1.070 and 1.419 mg/L; respectively. Toxicity of herbicides including metribuzin, thiobencarb, glyphosate and cryomazine showed different patterns of EC_{50}s (0.098, 9.903E-2, 18.220 and 132.256 mg/L; respectively), while those of insecticides; abamectin, chlorfluazuron, lambda cyhalothrin, amamectin benzoate and cypermethrin were 0.024, 0.035, 0.068, 0.107 and 2.535 mg/L; respectively. Based on EC_{50} values, *P. subcapitata* was more sensitive to the insecticide; abamectin and less sensitive to herbicide; cryomazine. The decreasing order of the sensitivity to green alga, in general, was as follows: abamectin> chlorfluazuron> mancozeb> lambda cyhalothrin> copper hydroxide > metribuzin> thiobencarb> amamectin benzoate> copper sulfate anhydrous> probiconazol> flusilazole> difenoconazole> cypermethrin> glyphosate> cryomazine.

Table (2): Differential Sensitivity of *P. subcapitata* to different pesticides.

Pesticide	EC_{50} (mg/L)	CL[a]	VL[b]	S[c]	Regression equation
Fungicides *Copper hydroxide*	0.093	(0.082- 0.105)	3.300E-1	4.427	Y= 4.567+ 4.427X
Copper sulfate	0.371	(0.262- 0.538)	1.583E-2	1.124	Y= 0.483+ 1.124X
Difenoconazole	1.419	(1.269- 1.593)	2.144E-2	1.876	Y= 1.876X- 0.285
Flusilazole	1.070	(0.909- 1.287)	4.131E-2	1.634	Y= 1.634X- 4.803
Mancozeb	0.044	(0.029-0.066)	1.394E-3	0.516	Y=0.699+ 0.516X
Probiconazole	0.578	(0.387- 0.902)	7.297E-3	0.896	Y= 0.213+ 0.896X
Herbicides: *Cryomazine*	132.256	(118.579- 147.558)	5.541E-2	2.513	Y= 2.513X-5.332
Glyphosate	18.220	(10.124 - 38.392)	1.660E-3	0.477	Y= 0.447X- 0.601
Metribuzin	0.098	(0.083- 0.117)	2.147E-2	1.629	Y= 1.629X+ 1.641
Thiobencarb	0.099	(0.075- 0.131)	8.518E-3	0.991	Y= 0.995+ 0.991X
***Insecticides**:* *Abamectin*	0.024	(0.015-0.036)	2.256E-3	0.596	Y=0.970+ 0.596X
Amamectin benzoate	0.107	(0.083-0.136)	2.447E-3	0.987	Y=5.484+ 0.987X
Chlorfluazuron	0.035	(0.029-0.043)	9.963E-3	1.463	Y=2.126+ 1.463X
lambda Cyhalothrin	0.068	(0.043-0.111)	8.138E-4	0.432	Y=0.432X+ 0.794
Cypermethrin	2.535	(1.975- 3.306)	7.780E-3	1.024	Y= 1.024X– 0.414

CL[a] : 95% Confidence Limits, VL[b] : Variance of slope, S[c] : Slope.

For each pesticide type (fungicide, herbicide and insecticide), toxicities and the RQs based on refinements of estimated and actual media exposures were ranked numerically from lowest toxicity or RQ to highest. Table 3 shows ranking of the tested pesticides based on their fold of toxicity and their estimated risk quotients. The results showed that, abamectin and chlorfluazuron were highly toxic (5510.66 and 3778.74 fold of toxicity; respectively) compared with cryomazine which was considered practically non-toxic to the tested alga.

Furthermore, data indicated that numerical ranking of toxicity was significantly correlated with the RQ ranking in most cases depending on the nature of the compound, bioavailability and concentrations of exposure. RQ values ranged from 0.591 to 27.769. Abamectin was the most toxic one and cryomazine was the lowest (RQ values were 27.769 and 0.832; respectively). Comparison of estimated RQ values with LOC for interpretation and analyzing potential risk to non-target organisms {for nonvascular plants (green algae or diatoms) and for short-term acute toxicity value less than 10 days, the LOC value is 1}, data disclosed that risk presumption is acute high risk when RQ, LOC equal 1.0 or exceed (as noticed for the effects of cypermethrin, flusilazole, probiconazole, copper sulfate, amamectin benzoate, thiobencarb and metribuzin). Additionally, an extremely high acute risk presumption (worst case risk) on aquatic organisms is suggested upon exposure to mancozeb, chlorfluazuron, lambda cyhalothrin and abamectin since RQs recorded 11.68, 14.83, 21.57 and 27.77; respectively). When LOC equal less than 1.0 (as noticed for cryomazine, glyphosate and copper hydroxide), risk presumption is acute endangered species.

Table (3): Toxicity rating and estimated risk quotient of the tested pesticides on *P.subcapitata.*

Pesticide	Fold of toxicity*	RQ
Cryomazine	Practically non-toxic	0.832
Glyphosate	7.23	0.835
Cypermethrin	52.17	2.988
Difenoconazole	93.20	0.982
Flusilazole	123.60	1.918
Probiconazol	228.82	1.153
Copper sulfate	356.48	1.582
Amamectin benzoate	1236.04	5.684
Thiobencarb	1335.51	1.167
Metribuzin	1349.55	2.306
Copper hydroxide	1422.13	0.591
lambda Cyhalothrin	1944.92	21.568
Mancozeb	3005.82	11.678
Chlorfluazuron	3778.74	14.833
Abamectin	5510.66	27.769

***Fold of toxicity is referred to the lowest toxicity value of cryomazine.**

DISCUSSION

Our current study points out possible risk presumptions due to exposure to different pesticide categories and the risk was expressed quantitatively as estimated RQ values. Our results strongly confirm the potential risk of pesticides on aquatic ecosystem. Lethal and effective concentrations tested in this study disclosed that even short exposures of freshwater ecosystem to pesticides concentrations in the µg-range might result in acute effects on unicellular chlorophyceae algae; *P. subcapitata* and might lead to a decrease of its populations. There are few literatures describing the ecological risk effects of pesticides on freshwater algae and comparative documents are rarely available.

Numerical ranking of toxicity was significantly correlated with the RQ ranking in most cases depending on the nature of the compound, bioavailability and concentrations of exposure. Based on RQ values (Table 3), risk presumption due to exposure of *P. subcapitata* to all tested insecticides represents the worst acute risk case (RQ= 2.988- 27.769 exceeding LOC) and hence a regulatory action may be warranted in addition to restricted use classification. The order of risk ranking based on estimated RQs is abamectin> lambda cyhalothrin> chlorfluazuron> amamectin benzoate> cypermethrin. The high toxicity observed in exposure to abamectin (as indicated in EC_{50} and RQ values) corroborates with the findings of Tisler and Erzen (2006). Furthermore, risk presumption due to amamectin benzoate exposure on *P. subcapitata* (Table 3) proved to be acute high risk (RQ= 5.684) when compared with LOC and such result agree with the findings of Garric et al., 2007. The ecological risk caused by abamectin is presumed to be more serious than by amamectin benzoate (Table 3). Although abamectin is not directly used in aquatic ecosystems, it may have adverse effects on aquatic biota either phytoplankton (Wislocki et al., 1989; Vieira, 2010) or zooplankton (Novelli, 2010) through direct application as well as via runoff from experimentally contaminated plots. Additionally, ivermectin compounds showed worst case risk presumptions (RQ= 1.05–36.2) and many efforts were made to prevent repeated entry in aquatic environments and thus its accumulation in sediments (Garric et al., 2007).

Fungicides were also presumed to pose a high acute risk on aquatic ecosystem but in a trend lower than insecticides and this may be mitigated through restricted use classification. Four compounds out of six tested fungicides recorded RQ values exceeding LOC. The order of risk ranking based on estimated RQs is mancozeb> flusilazole> copper sulfate> probiconazole> difenoconazole> copper hydroxide. Both mancozeb and flusilazole are presumed to pose a high acute risk on aquatic ecosystem (RQ=11.678 and 1.918; respectively exceeding LOC) and such findings are strengthened by USEPA where both compounds were considered as algistatic, rather than algicidal on *P. subcapitata* based on the respective studies (USEPA, 2005). Furthermore, it’s worthy to mention that although the toxicity of copper hydroxide is higher than toxicity of copper sulfate (≈ 3 times fold), but estimated RQ values of copper sulfate were higher than for copper hydroxide. Comparative studies; regarding only EC_{50} toxicity value, reported the toxic effect of copper sulphate (Gregor et al., 2008) and copper oxide nano particles (Aruoja et al., 2009) on *P. subcapitata.* The toxicity of copper oxides was due to the solubilized bioavailable fraction, most likely Cu^{2+} ions. The bioavailable EC_{50} values of copper oxides (0.71 mg/ L) were significantly different from the EC_{50} of copper sulphate (0.02 mg/L).

Among tested herbicides, only metribuzin and thiobencarb are presumed to pose a high acute risk on aquatic ecosystem (RQ=2.306 and 1.167; respectively exceeding LOC). The order of risk ranking based on estimated RQs is metribuzin> thiobencarb> glyphosate> cryomazine. Our data are consistent with other studies where both thiobencarb and metribuzin were toxic to *P. subcapitata* with a 120-hr EC_{50} of 17 µg/ L and 0.043 µg/ mL; respectively (EFED-EPA, 2009; Choi et al., 2012). In other situation, both acute exposure and RQ were estimated for metribuzin (20.815 ppb and 5.215; respectively) whereas, these estimates recorded 17 ppb and 5.8553; respectively for thiobencarb (Peterson, 2006). On the other hand, when LOC equal less than 1.0 (as noticed for glyphosate) risk presumption is acute endangered species and a regulatory action may be warranted. Comparatively, acute toxicity of formulated products containing glyphosate tested on freshwater *P. subcapitata* indicated that glyphosate is generally less toxic to aquatic vascular plants than to algae (EC_{50}= 12.5 ppm)(Patterson, 2004).

CONCLUSION

The results reported herein support the hypothesis that numerical ranking of RQs for the purpose of comparing potential ecological risks of pesticides is a valid approach. Ranking based only on toxicity was statistically correlated with the RQ estimates except in few cases. Therefore, ranking of ecological risk based solely on toxicity should not be used or should be used with caution. Ranking among pesticides based on RQs, especially for acute risks, should largely be used and interpreted based on refined environmental exposure estimates and actual values. Accordingly, the use of pesticides to control pests should be accompanied by efforts to limit, as far as possible, the contamination of aquatic ecosystems.

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مقارنة المخاطر البيئية للمبيدات على طحلب المياه العذبة الأخضر وحيد الخلية *Pseudokirchneriella subcapitata* بأستخدام منهج تصنيفى لمحصلة الخطر.

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تستخدم عمليات تقييم الخطر البيئى غالبا طريقة محصلة الخطر (RQ) لوصف الخطر كميا. وقد تم تقدير تأثيرات سمية المبيدات (وتضم ٥ مبيدات فطرية- ٤ مبيدات حشائش- ٦ مبيدات حشرية وكلها تجهيزية) على طحلب المياه العذبة الأخضر *Pseudokirchneriella subcapitata*. وفى هذا السياق تم أجراء أختبارات السمية الحادة لمدة ٩٦ ساعة لكل مبيد وقد تم تقييم قيم محددات السمية وقيم محصلة الخطر اعتمادا على تهذيب تركيزات التعرض. وقد تم عرض أفتراضات الخطر متوازية مع مستوى الأهمية المقابل(LOC) . وقد أظهرت نتائج السمية ترتيب رقمى تفاضلى واضح داخل كل فئة من المبيدات. فيما يخص مجموعة المبيدات الفطرية فقد وجد أن مركبmancozeb هو الأكثر سمية و مركب difenoconazole هو الأقل سمية فى هذه المجموعة (٣٠٠٦ و ٩٣ ضعف السمية على التوالى). أما بالنسبة لمجموعة مبيدات الحشائش فقد وجد أن مركبmetribuzin هو الأكثر سمية (1349ضعف السمية) بينما وجد أن مركب cryomazine يعد غير سام عمليا. علاوة على ذلك فقد أثبت مركب abamectin أنه أشد المبيدات الحشرية سمية (٥٥١١ ضعف السمية). وقد وجد أن الترتيب العددى المعتمد على السمية يرتبط معنويا فى معظم الأحوال بطريقة الترتيب المستخدم لمحصلة الخطر ويعتمد على طبيعة المركب و الأتاحة الحيوية وتركيزات التعرض. وقد أثبتت أفتراضات الخطر المصاحبة ان مركبات abamectin, lambda-cyhalothrin, chlorfluazuron and mancozeb تسبب أسوء حالات الخطر الحاد للكائنات الغير مستهدفة فى البيئة المائية وبالتبعية يجب أتخاذ أجراءات تنظيمية بالأضافة الى الحد من أستخدامها (RQs=27.769; 21.568; 14.833 and 11.678 علي التوالى متجاوزة قيمة LOC). ووفقا لقيم محصلة الخطر فأن أفتراضات الخطر نتيجة تعرض طحلب *P. subcapitata* لكل المبيدات الحشرية المختبرة تمثل أعلى أحتمالية للخطر الحاد (RQ= 2.988- 27.769 متجاوزة قيمة LOC). كذلك فان المبيدات الفطرية يفترض أنها تسبب خطرا عاليا على البيئة المائية لكن بنمط أقل مما تسببه المبيات الحشرية. من بين مبيدات الحشائش فان مركبات thiobencarb&metribuzin فقط يفترض أنها تسبب خطرا حادا عاليا على البيئة المائية (RQ= 2.306 and 1.167 متجاوزة قيمة LOC). وفى الخلاصة فأن نتائج هذه الدراسة تؤيد نظرية أستخدام الترتيب العددى لمحصلة الخطر بغرض مقارنة المخاطر البيئية المحتملة للمبيدات ويمكن لهذه البيانات أن تضيف الى قاعدة البيانات الحالية للمبيدات فيما يتعلق بالتأثيرات السلبية للمبيدات على البيئة المائية.

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