Universidade de Aveiro Departamento de Química
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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Métodos Biomoleculares Avançados, realizada sob a orientação científica de Fernando Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro.

Dedico este trabalho à minha Mãe, ao meu Pai, à Ana e à Aurora

o júri

presidente	Maria Lourdes Pereira Professora Associada com agregação Universidade de Aveiro
vogais	Ulisses Miranda Azeiteiro Professor Auxiliar Universidade Aberta
	Fernando José Mendes Gonçalves Professor Associado com agregação Universidade de Aveiro

agradecimentos

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Aos meus amigos e amigas, porque me são essenciais !
(e porque não cabem todos aqui...)

Uma atenção especial para o meu Pai, a minha Mãe, a minha irmã e à Aurora pelo contínuo e fundamental apoio com que sempre pude contar!
À minha Tia Madrinha por todas as ajudas que me prestou!
À minha família !

Aos dois lá de casa, Sofia e Coelho, obrigado por tudo :)
À Miriam e companhia, obrigadeinha !
Ao Spok
Ao Mac

palavras-chave

Toxicologia, *Daphnia*, Pesticidas Organofosforados, Testes Agudos e Crónicos, Autóctone.

resumo

O uso abusivo de pesticidas pode ter consequências graves para o ambiente aquático. É importante conhecer e divulgar os perigos que esse abuso pode causar em muitos ecossistemas.
A resposta de alguns organismos a estas substâncias, é muitas vezes uma das primeiras ferramentas utilizadas na análise do efeito que um determinado agente xenobiótico pode causar no ambiente aquático.

Neste estudo, foram observados os efeitos agudos e crónicos causados pelos pesticidas Quirlan® (fórmula comercial de Chlorfenvinphos) e Kimlux® (fórmula comercial de Quinalphos), em diferentes parâmetros de reprodução e crescimento das espécies *Daphnia magna* (espécie padrão) e *Daphnia longispina* (espécie autóctone).

Os efeitos agudos e crónicos foram significativos em ambas as espécies e para ambos os pesticidas, e as taxas de crescimento intrínseco foram significativamente afectadas nas concentrações mais elevadas.

Embora haja alguma falta de informação a respeito da concentração destes pesticidas no ambiente, estes revelaram (em algumas concentrações) a sua toxicidade. É, por isso, possível que uma exposição crónica a concentrações ainda inferiores destes pesticidas, possa levar à manifestação de efeitos significativos a nível do crescimento e reprodução dos indivíduos, sendo, numa fase seguinte, o ecossistema afectado.

keywords

Toxicology, *Daphnia*, Organophosphorous Pesticides, Acute and Chronic Tests, Autochthonous.

abstract

The uncontrolled use of pesticides can lead to drastic consequences to the aquatic environment. It is important to inform the community of the dangerous effects that an abuse in pesticides may cause to many ecosystems.
The response of many organisms to these substances is often one of the first tools that can be used to evaluate the effect, and the potential risk of a given xenobiotic to the whole aquatic environment.

In this study, the acute and chronic effects of the organophosphorous pesticides Quirlan® (commercial formulation of Chlorfenvinphos) and Kimlux® (a commercial formulation of Quinalphos) were observed in growth and in reproduction of *Daphnia magna* (a standard testing species) and *Daphnia longispina* (an autochthonous species).

Acute and chronic exposures caused significant effects in the two *Daphnia* species. The Intrisic Growth Rate presented significant differences in both species with both tested pesticides.

Although some absence of information exists on the environmental concentration and effects of Chlorfenvinphos and Quinalphos, this study revealed their toxicity in some of the tested concentrations. Thus, it is likely that a chronic exposure to even lower concentrations of these pesticides can lead to significant effects in individual-level growth and reproduction characteristics, and, at a later stage, to problems within the ecosystem.

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Introduction

The protection of aquatic ecosystems, from the adverse effects of chemical pollution caused by human action, is of outmost importance, given its environmental, economical and genetic values. In order to study and evaluate such problems in a long-term basis, we can observe the physical, biochemical, and other responses from the organisms that inhabit these ecosystems.

PESTICIDE USE: RELEVANCE AND RISK

In order to cope with the exponential growth of the human population, *versus* the scarcity of food, efforts should be aimed towards adequate agriculture and livestock production (Kituyi et al., 1997; Marques, 2003; Goncalves and Alpendurada, 2004), and for this matter, pesticides have become a key product. The chemical treatment on fields made it a lot easier for farmers to control many kinds of threats, beating the efficiency of both manual and mechanic treatments (Spliid and Koppen, 1998). In fact, during most of the 20th century, the use of pesticides has become more and more significant for pest control of weed, insects and fungi (Guilhermino et al., 1996a; Spliid and Koppen, 1998) and of course that great productivity gains were achieved.

FROM APPLICATION TO CONSUMPTION

Agricultural and cultivated soils are the major reservoir of environmental pollutants (e.g., pesticides), and therefore they represent a source from which residues can, often undesirably, be released to the atmosphere and water bodies, and in case these residues become biologically available (i.e. bioavailable), they can contaminate living organisms, specially when used inappropriately by farmers (Goncalves and Alpendurada, 2004). It is the retention, transformation and transport processes, as well as their interactions, that direct the fate of a pesticide in the soil (Gamón et al., 2003).

It has been reported that approximately 90% of agricultural pesticide applications never reaches their target organisms (Roast et al., 1999). In many aspects, the greatest potential for adverse effects of pesticides is through contamination of the hydrologic system, which supports aquatic life as well as related food chains (Gilliom, 2001). The use of pesticides in agriculture can lead to both surface and ground water contamination either by drift, runoff, drainage and leaching (Cerejeira et al., 2003) as well as direct application to treat freshwater crops (Fisher et al., 2000). Runoff losses at the edge of fields may reach several percent of the amount of applied pesticides, and concentrations can get up to several mg/l if runoff occurs soon after application. Nonetheless, these concentrations may be swiftly attenuated in the transport system by dilution, deposition or trapping of sediments along the flow path (Kreuger, 1998). Together with the runoff water, suspended soil can also be introduced into the water body, carrying considerable portions of absorbed pesticides (Liess et al., 1999; Gamón et al., 2003). It has been reported that the loss of insecticides as a consequence of runoff events reached percentages from 0.1% to 5% of the total amount applied on the field (Liess et al., 1999; Capel et al., 2001; Gilliom, 2001). In fact, a high organic matter input from the surroundings, usually characterizes water bodies that are close to intensively cultivated areas.

The amount of pesticides that will be found in the water, will also depend on the time period between its application in the field and the rain event, the maximum precipitation and various other soil parameters (Liess et al., 1999). Physico-chemical characteristics play an important role in determining the mobility of a chemical in the environment. This way, substances with higher water solubility are generally introduced through soil filtration, and are lost at greater rates than substances that are moderately soluble, which are carried by water runoff during heavy rains (Kreuger, 1998; Liess et al., 1999; Naddy and Klaine, 2001). Toxicity itself depends on both exposure concentration and duration, and in this case, more hydrophilic pesticides tend to metabolize faster and to cause less acute toxicity (Naddy and Klaine, 2001).

Surface water contamination may have ecotoxicological effects for aquatic flora and fauna, as well as for human health if this water is used for public consumption (or indirectly by feeding on organisms who had contact with it, i.e., fishes), recreation or many other purposes (Liess et al., 1999; Gilliom, 2001; Abrantes et al., in press). The degree of contamination is usually dependant on the agricultural season and often does not last for long periods. On the other hand, ground water contamination may lead to a continuous human exposure, as it represents the most important source of drinking water supply in many countries. In Portugal, 53% of the drinking water is extracted from ground water (Cerejeira et al., 2003). One must also have in mind that contaminated ground water bodies can also contaminate surface waters.

There are other ways for humans to get exposed to pesticides, a recent study focusing on the quantification of pesticides in vegetables - for human ingestion - detected measurable amounts of residues in 100% of the 60 samples of six seasonal vegetables (Kumari et al., 2002).

PESTICIDE USE: QUANTITIES AND QUALITIES

According to the 2002 Phytopharmaceutical Product Sales Report by the Portuguese DGPC (Vieira, 2004), 15,501,379 Kg of phytopharmaceutical products (expressed in active substance) were sold in Portugal during the year 2001, from which 254,791 Kg were organophosphorous compounds (OPs). The same source revealed that in the following year (2002) there was a 12.6% (1,949,556 Kg) increase in the total of phytopharmaceutical products sold, up to 17,450,935 Kg, while OP sales increased 14.2% (36,128 Kg) up to 290,664 Kg.

ORGANOPHOSPHOROUS INSECTICIDES

Organophosphorous Insecticides have been widely used from the 1930s until the present days (Guilhermino et al., 1996b; Sogorb and Vilanova, 2002; Ferrari et al., 2004), applied in agricultural fields, forests and other places, in order to control the quantity of several kinds of pests (Naddy and Klaine, 2001), this way becoming, along with carbamates, the most widely used class of insecticides in the world, replacing the persistent and problematic organochlorine compounds. They were initially successful given their fast degradation and high toxicity (Ferrari et al., 2004), and because apparently, they did not accumulate in food chains (Guilhermino et al., 1996a). They tend, however, to be less specific (Papp et al., 2004), a fact that can lead to the development of serious problems at the population level, like certain aquatic species (mainly invertebrates) that are affected by these products, while not being its initial target (Barata et al., 2004), and thus, OPs may represent a major toxicological hazard when released in the environment (Papp et al., 2004).

The organophosphorous insecticides of major commercial and toxicological interest are esters or thiols derived from phosphoric, phosphonic, phosphinic or phosphoramidic acid and almost all of them inhibit cholinesterase enzymes. Some authors support that OPs are biodegraded in mammals, and consequently are less toxic (Sogorb and Vilanova, 2002), but they agree with many who defend that the effect of OPs in humans undoubtedly is a cause for concern. This is because anticholinesterase OPs may cause acute effects, and exert several forms of toxicity (Ray and Richards, 2001), with symptoms generally occurring with 50% acetylcholinesterase inhibition, such as headache, exhaustion, sweating, chest tightness, mental confusion, blurred vision, muscle twitching and abdominal cramps (PSD, 2005). Higher exposure may cause neurotoxicity (Ray and Richards, 2001; Jamal and Julu, 2002), immunotoxicity (Galloway and Handy, 2003) and even death (Hsieh et al., 2001). The detoxification from OPs usually occurs through oxidation and hydrolysis (Sogorb and Vilanova, 2002).

OPs can, and should be, carefully selected based on their chemical structure, to ensure a high efficiency against their target pest, while controlling the risk to non-target species by following precautions (PSD, 2005).

QUINALPHOS AND CHLORFENVINPHOS

In this study, both Quinalphos and Chlorfenvinphos were used in their commercial formulations (molecular structures represented in Figs I.1. and I.2., respectively). Both these chemicals are organophosphorous insecticides, and were, during 2004, amongst the most sold pesticides in the centre of Portugal.

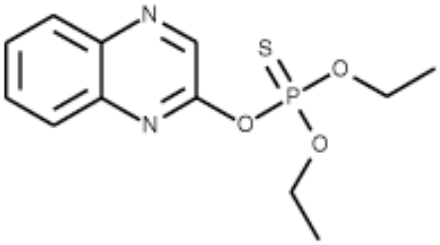

Fig I.1. – Molecular structure of Quinalphos [O,O-diethyl O-quinoxalin-2-yl phosphorothioate]

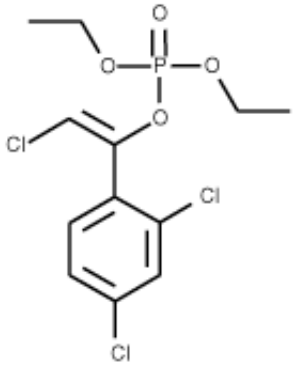

Fig I.2. – Molecular structure of Chlorfenvinphos [2-chlorophenyl ethenyl diethyl phosphate]

Quinalphos [O,O-diethyl O-quinoxalin-2-yl phosphorothioate] is considered to be moderately hazardous by the WHO (2002). It is an environmental oestrogenic organophosphorous insecticide pollutant, that some studies have detected in vegetables and soils (Kumari et al., 2002), persisting in some cases with a half-life of 2 weeks (Babu et al., 1998). Recent studies have shown that quinalphos can induce micronucleus and chromosomal aberrations in bone marrow cell, as well as a high incidence of abnormal sperm in mice, following oral exposure to this xenobiotic. Decreased sperm motility and sperm count, increased percentage of sperm abnormalities and testicular tissue damage, lethargy,

staggering during locomotion, weight loss and even death, were noticed (Babu et al., 1998; Debnath and Mandal, 2000; Pant and Srivastava, 2003).

Chlorfenvinphos [2-chlorophenyl ethenyl diethyl phosphate] is considered highly hazardous by the WHO (2002). According to DGPC (2004) this chemical is very dangerous to aquatic organisms, and water contamination should therefore be prevented by not using this substance near watercourses. This pesticide is, however, one of the most frequently applied in the Portuguese paddies (Pereira et al., 2000), fact that is supported by its high trade volume (Vieira, 2004). Some of Chlorfenvinphos´ effects have already been studied, and it has been reported that it can cause serious health effects in humans, and surprisingly its presence has even been reported in milk (Kituyi et al., 1997). The bioaccumulation ability of chlorfenvinphos in living tissues represents a potential environmental risk to many different organisms (Serrano et al., 1997).

AQUATIC ECOTOXICOLOGY: *DAPHNIA* SP. AS A TEST SPECIES

It is common practice to perform ecotoxicological tests to evaluate the effect of certain chemicals in the environment (ASTM, 1980). *Daphnia* spp. (Cladocera; Branchiopoda; Crustacea – see Fig I.3.) is one of the most widespread zooplanktonic crustaceans in the world, and play a central role in the food webs of pelagic freshwater communities (Vega and Pizarro, 2000). For this reason, but also because it is a non-target species inhabiting many freshwater ecosystems, *Daphnia* sp. keeps being widely used in laboratory as a standard test organism in aquatic toxicology (Barata et al., 2004). Klein (2000) states that biological tests with *Daphnia magna* Straus have been used and accepted throughout the world during the last decades, as instruments for the estimation of the acute and chronic toxicity of xenobiotics in aquatic environments. Actually, standard methods to conduct this type of tests with *Daphnia* spp. have also been established (OCDE, 1996; 2000).

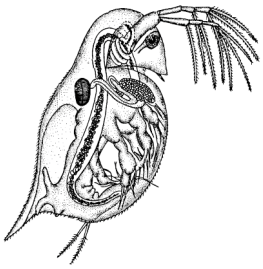

Fig I.3. – Schematic representation of an organism from the genus *Daphnia*

The high sensitivity of Daphnids to organophosphorous pesticides (Guilhermino et al., 1996a; Naddy et al., 2000; Barata et al., 2001), and because they usually inhabit water bodies near agricultural fields (where OP pesticide treatments are frequently applied) make them widely used in aquatic risk assessment (Barata et al., 2004), in particular, for ecotoxicity evaluations in which this research is contextualized.

Daphnia longispina belongs to a complex of many species (i.e., *D. ambigua, D. hyalina, D. galeata, D. cucullata*) and was used in the present study, because it is an autochthonous species, having been found in several different places in Portugal [i.e., lakes Vela, Braça, Mira and Tapada Grande (Antunes et al., 2003)]. Being smaller organisms than *D. magna*, differences in pesticide resistance are expected due to the phenotypic plasticity and different body proportions, and these relationships would be interesting to evaluate.

There are great advantages in using these species: identical animals can be bred through acyclic parthenogenesis, enabling a strong genetic homogeneity: it allows the use of low culture medium volumes, it has a short life cycle, and it is easy to maintain (Barata et al., 2000; Klein, 2000; Guilhermino et al., 2000). Regarding laboratory culture maintenance, *D. longispina* is very similar to *D. magna* (Antunes, 2001).

AQUATIC ECOTOXICOLOGY: ACUTE AND CHRONIC BIOASSAYS

Model bioassays that are used to evaluate the effects of hazardous chemicals on aquatic organisms usually include acute tests and chronic tests (Sanchez et al., 1999), and *Daphnia* spp. are among the most used organisms.

Acute tests usually are the starting point. These tests use higher xenobiotic concentrations (lethal concentrations), and for that fact, they are typically of shorter duration than chronic tests. In this case, the measured parameter is the animals' death (mortality). These tests allow, among other conclusions, the calculation of indexes like the LC_{50} – median lethal concentration – that represents the exposure concentration at which the death of 50% of the animals is expected to happen in a given period, in this case, 48 hours (48h-LC_{50}). This presents a fast report of the toxicity caused by the xenobiotic, which is very important for the design and development of further testing.

Chronic tests use lower xenobiotic concentrations (sub-lethal concentrations), and they usually last longer, so that one can monitor and evaluate the effect of these chemicals in parameters such as the variation in normal reproduction, growth or other effects along a timeline. This way, substantial information about these organisms' adaptation is acquired (Guilhermino et al., 1999).

Objectives

The essential objectives of this study were:

- To examine the acute and chronic effects caused by Quinalphos while in a commercial formulation in both *D. magna* (standard species) and *D. longispina* (autochthonous species).

- To examine the acute and chronic effects caused by Chlorfenvinphos while in a commercial formulation in both *D. magna* and *D. longispina*.

- To compare the responses of *D. magna* and *D. longispina* to the above-mentioned xenobiotics.

ACUTE AND CHRONIC EFFECTS OF KIMLUX® (COMMERCIAL FORMULATION OF QUINALPHOS) ON THE LIFE-HISTORY PARAMETERS OF *DAPHNIA MAGNA* AND *D. LONGISPINA*.

MESQUITA, N. M., GONÇALVES, F.

Abstract

The protection of aquatic ecosystems from the adverse effects of chemical pollution caused by human action is of outmost importance, given its biological, environmental, economical and genetic values. In order to study and evaluate long-term problems and effects, we can observe the physical and biochemical responses of the organisms that inhabit these ecosystems. In this work, the acute and chronic effects of the organophosphorous pesticide Kimlux® (a commercial formulation of Quinalphos), in growth and reproduction of both *Daphnia magna* (standard testing species) and *Daphnia longispina* (autochthonous species) was investigated. *D. magna* showed higher acute tolerance to this pesticide (48h EC_{50} = 0.586 µg/L) than *D. longispina* (48h EC_{50} = 0.197 µg/L), and in the chronic exposure tests, both species were affected by some of the tested concentrations. There isn't much information on the concentration and effects of Quinalphos, however, this study revealed its toxicity in some of the tested concentrations. Thus, it is likely that a chronic exposure to even lower concentrations of this pesticide can lead to significant effects in individual-level growth and reproduction parameters.

Introduction

The protection of aquatic ecosystems from the adverse effects of chemical pollution caused by human action is of outmost importance, given its biological, environmental, economical and genetic values. In order to study and evaluate long-term problems and effects, we can observe the physical and biochemical responses of the organisms that inhabit these ecosystems.

In order to cope with the exponential growth of the human population versus the scarcity of food, efforts should be aimed towards adequate agriculture and livestock production (Kituyi et al., 1997; Marques, 2003; Goncalves and Alpendurada, 2004), and for this matter, pesticides are a key product. The chemical treatment on fields, made it a lot easier for farmers to control many kinds of threats, beating the efficiency of both manual and mechanic treatments (Spliid and Koppen, 1998). The use of pesticides has become more and more significant for pest control of weed, insects and fungi during most of the 20th century (Guilhermino et al., 1996; Spliid and Koppen, 1998) and great productivity gains were subsequently achieved.

Surface water contamination may have ecotoxicological effects for aquatic flora and fauna, as well as for human health if this water is used for public consumption, recreation or many other purposes (Liess et al., 1999; Gilliom, 2001). The contamination is usually temporary, and the degree depends on many factors (e.g., agricultural season, weather, and the way the pesticide is applied). If the planktonic communities, which belong to the first levels of the food chain, are affected by pesticide contamination, upper levels from the food chain will most probably be compromised as well, which may cause severe effects to the ecosystem.

According to the 2002 Phytopharmaceutical Product Sales Report by the Portuguese DGPC (Vieira, 2004), 15,501,379 Kg of phytopharmaceutical products (expressed in active substance) were sold in Portugal during the year 2001, from which 254,791 Kg were organophosphorous compounds (OP). The same source revealed that in the following year (2002) there was a 12.6% (1,949,556 Kg)

increase in the total of phytopharmaceutical products sold, up to 17,450,935 Kg, while OP sales increased 14.2% (36,128 Kg) up to 290,664 Kg.

Organophosphorous insecticides have been widely used from the 1930s until the present days (Guilhermino et al., 1996; Sogorb and Vilanova, 2002; Ferrari et al., 2004), being applied in agricultural fields, forests and other places, in order to control several kinds of pests (Naddy and Klaine, 2001) and this way becoming, along with carbamates, the most widely used class of insecticides in the world, in someway replacing the persistent and problematic organochlorine compounds. They were initially successful because of their fast degradation and high toxicity (Ferrari et al., 2004), and also because apparently, they did not accumulate in food chains (Guilhermino et al., 1996). They tend, however, to be less specific (Papp et al., 2004), and certain non target species, like aquatic invertebrates, can be affected by these products (Barata et al., 2004). Therefore, these pesticides can represent a major toxicological hazard when released to the environment (Papp et al., 2004).

The organophosphorous insecticides of major commercial and toxicological interest are esters or thiols derived from phosphoric, phosphonic, phosphinic or phosphoramidic acid and almost all of them inhibit cholinesterase enzymes. Some authors support that OPs are biodegraded in mammals, therefore being less toxic (Sogorb and Vilanova, 2002), but they agree with many who defend that the effect of OPs undoubtedly is a cause for concern. Therefore OPs can, and should be carefully selected based on their chemical structure, to ensure a high efficiency against their target pest, while controlling the risk to other species by following precautions (PSD, 2005).

A commercial formulation - Kimlux® - with Quinalphos (molecular structure represented in Fig II.1.) as active ingredient, was used in this work. Quinalphos was one of the most sold OP pesticides during 2002, in the central western coast of Portugal. Teixeira et al. (2004) found that 30 people were killed or clinically intoxicated by Quinalphos, from 2000 to the end of 2002 (only Paraquat® intoxicated more people, in this case 31), in the centre of Portugal.

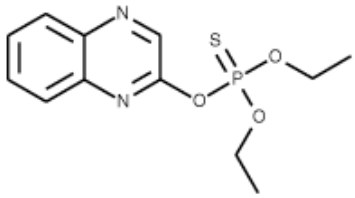

Fig II.1. – Molecular structure of Quinalphos
[O,O-diethyl O-quinoxalin-2-yl phosphorothioate]

Quinalphos is an environmental oestrogenic organophosphorous insecticide pollutant, considered moderately hazardous by the WHO (2002).

Daphnia spp. (Cladocera; Branchiopoda; Crustacea) are one of the most widespread zooplanktonic crustaceans in the world, and play a central role in the food webs of pelagic freshwater communities (Vega and Pizarro, 2000). Its high sensitivity to organophosphorous pesticides (Guilhermino et al., 1996; Naddy et al., 2000; Barata et al., 2001), along with the fact that it often inhabits water bodies near agricultural fields where OP pesticide treatments are more frequently applied, makes it widely used in aquatic risk assessment (Barata et al., 2004).

Daphnia longispina belongs to a complex of many species (i.e. *D. ambigua, D. hyalina, D. galeata, D. cucullata*) and was used in the present study, because it is an autochthonous species, having been found in several different places in Portugal [e.g., lakes Vela, Braça, Mira and Tapada Grande (Antunes et al., 2003)].

The objective of this study was to examine the acute and chronic effects caused by Quinalphos while in its commercial formulation (Kimlux®), in both *D. magna* and an autochtonous clone of *D. longispina*. The main emphasis was to evaluate the toxicological responses of both species in order to determine if *D. magna* is representative of local cladocerans.

Material and Methods

STOCK CULTURE OF DAPHNIDS

Parent individuals of *Daphnia magna* Straus clone A [*sensu* Baird et al. (1989)] and *Daphnia longispina* O. F. Müller clone EM7 [*sensu* Antunes et al. (2003)] were both reared in 800 ml of ASTM hard water (ASTM, 1980; EPA, 1989) and fed every two days with *Pseudokirchneriella subcapitata* Korshikov, maintained in our laboratory as described by Stein (1973), in a concentration of 3 x 10^5 cell.ml^{-1} for *D. magna* (OECD, 2000); and 1.5 x 10^5 cell.ml^{-1} for *D. longispina* (Antunes et al., 2003). An organic additive made of *Ascophyllum nodosum* (L.) Le Joli seaweed extract (Baird et al., 1988) was prepared by dilution of a stock solution, and added to the culture medium to a final concentration of 6 ml/L (Soares, 1989). The cultures were maintained in a semi-static system, with a photoperiod of 16^L:8^D and a temperature of 20±1 ºC.

CHEMICALS AND TEST SOLUTIONS

Kimlux® pesticide was acquired in its commercial formulation, with specifications according to Table II.1..

Table II.1. – Pesticide Description

Commercial Name	Active Ingredient	A.I. Concentration
Kimlux®	Quinalphos	243 g/L

Stock solutions were obtained by dilution of the original products in ultrapure water. The volumes used in each test were quite small, allowing the dilution with ultrapure water instead of the original culture medium (i.e., ASTM Hard Water) thus preventing the occurrence of reactions that could change its properties, prior to the testing period. These solutions were stored at 2-8ºC in

dark glass bottles. No significant changes in pH were noticed in the highest concentrations of this pesticide, which was kept in the range of 6 – 9 pH units.

EXPERIMENTAL DESIGN

ACUTE IMMOBILIZATION TEST

This test was conducted according to OECD's guidelines for the *Daphnia* sp. Acute immobilization test (OECD, 2000), using a single clone of *D. magna* and a single clone of *D. longispina*. Only neonates from the third to the fifth brood with less than 24h were used in these experiments to minimize maternal effects.

Pesticide stock solutions were prepared previous to each test, by dilution in ultrapure water, and kept in the dark and in cold storage. Daphnids were maintained in groups of 5, for a period of 48 hours in glass vessels, with 100 ml of test solutions, with four replicates. The test conditions were similar to those described for the parents´ culture, but neither food nor extract were administrated during the 48-hour period, in order to minimize test variables. Both dissolved oxygen and pH were measured (using the WTW Oxi 330 and the WTW pH 330 meters, respectively) in the beginning and in the end of each test. After the 48-hour period, the number of organisms that remained immotile for approximately 15 seconds after a smooth agitation of the vessel was recorded.

CHRONIC GROWTH AND REPRODUCTION TEST

This test was conducted according to EPA's guidelines for *Ceriodaphnia dubia* Survival and Reproduction Test – Method 1002.0 (EPA, 1989) - adapted for testing with both *D. magna* and *D. longispina*. In these experiments, same species and clones were used. Again, in order to minimize maternal effects, only third to fifth brood neonates, with less than 24 hours, were used.

Pesticide stock solutions were prepared previously to each test, by dilution in ultrapure water, and kept in the dark, and in a cold storage. For this test, ten

replicates (for the control and for each of six different concentrations) were prepared in 50 ml glass vessels, and filled with the appropriate volumes of ASTM and test pesticide (from stock solution). The tested concentrations were different for each species (Table II.2.).

Table II.2. – Pesticide concentration in each group, for each species

	D. magna	***D. longispina***
Group	Concentration (µg/l)	Concentration (µg/l)
C	0.0000	0.0000
1	0.0250	0.0250
2	0.0375	0.0330
3	0.0563	0.0436
4	0.0844	0.0575
5	0.1266	0.0759
6	0.1898	0.1002

Daphnids were individually cultured in each vessel, with the same conditions described for the parents' culture, except that all animals were fed every single day, and were transferred to newly prepared vessels, once every two days. Both oxygen concentration and pH were measured at least once a week to ensure that they were not limiting factors for biological responses. The test was to end after all females released the 3rd brood, or died, to a maximum duration of 15 days.

During the test period, several parameters were observed and recorded. All females were measured three times: in the beginning of the test, after releasing the 1st brood and in the end of the test. The females' moult is usually lost from its body after all neonates have been released, and for this matter, the length of first exopodite (EL) of the second antennae (Fig. II.2.), from each moult, was measured to calculate the female organism's body length in millimeters (equations II.1. and II.2.). This was accomplished because of the allometric relationship that was described between these two variables (Pereira et al., 2004).

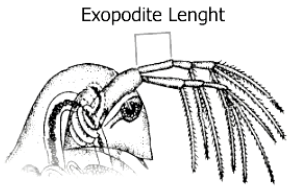

Fig II.2. – Schematic representation of the correct length measurement of the 1st exopodite of the 2nd antenna (EL)

$BL_{D.\ magna} = 10.499 \times EL_{D.\ magna} - 0.329$ (mm) ($r^2 = 0.9392$) (equation II.1.)

$BL_{D.\ longispina} = 10.660 \times EL_{D.\ longispina} - 0.186$ (mm) ($r^2 = 0.9656$) (equation II.2.)

The daily growth rate (DGR) was then calculated (equation II.3.), with BL_f standing for the organism's final body length (mm), BL_i standing for the organism's initial body length (mm) and Δt for the time interval (days) (Burns, 2000).

$$DGR = \frac{\ln(BL_f) - \ln(BL_i)}{\Delta t} \quad (days^{-1}) \qquad \text{(equation II.3.)}$$

The age (days) of each female at each brood, together with the number of offspring, were recorded for each of the 3 broods. Five random first-brood neonates from each vessel were measured for the total body length, this meaning from the top of the head to the base of the spine (Fig. II.3.). All measurements were done using an Olympus SZX9 Stereomicroscope with an ocular micrometer.

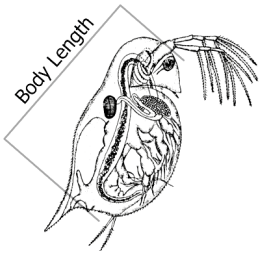

Fig II.3. – Schematic representation of the measurement of the Total Body Length (BL)

The intrinsic growth rate (r, day^{-1}) is one of the more relevant ecotoxicological endpoints, which provide us with relevant measurements of ecological impact (Stibor and Lampert, 1993; Trubetskova and Lampert, 2002). It was iteratively calculated using the Euler-Lotka equation (equation II.4.), where x stands for the age class (in days; 0 to n), l_x is the female's survival probability at day x, and m_x represents the fecundity at day x.

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x \qquad \text{(equation II.4.)}$$

All neonates that were released in the same day as the female's death were also counted, as they influence further population growth. Standard deviation for this test was calculated according to the Jackknife technique (Meyer et al., 1986).

DATA ANALISYS

ACUTE IMMOBILIZATION TEST

The number of immobilized animals was plotted against the concentration, to calculate the organism's 48-hour EC_{50} value for the given pesticide at a 95% confidence limit, with the use of Probit Analysis (Finney, 1971).

CHRONIC GROWTH AND REPRODUCTION TEST

The statistical comparison, for each assessed variable, was achieved with the use of one-way ANOVAs, followed by post-hoc Dunnett's tests, to compare results from each treatment and the control (Zar, 1996). In all statistical tests, the level of significance was set at 5%.

The mortality caused by the tested conditions was analyzed using Fisher's Exact Test (EPA, 1989). Both NOEC (No Observed Effect Concentration) and LOEC (Low Observed Effect Concentration) for some of the reproduction and growth parameters were also calculated with the use of one-way ANOVAs, followed by post-hoc Dunnett's tests.

Results

ACUTE TOXICITY TEST

At the end of the 48-hour test, the EC_{50} determined for *D. longispina* (0.1975 µg/L) was about 2.5 times smaller than the value determined for *D. magna* (0.5863 µg/L) – see Table II.3..

Table II.3. – 48-hour EC_{50} values, with 95% confidence limits (CL), for *D. magna* and *D. longispina* exposed to Kimlux® (n=20)

D. magna		*D. longispina*	
EC_{50} = 0.5863 µg/L		EC_{50} = 0.1975 µg/L	
Lower CL	Upper CL	Lower CL	Upper CL
0.5237 µg/L	0.6671 µg/L	0.1802 µg/L	0.2213 µg/L

CHRONIC GROWTH AND REPRODUCTION TEST

Table II.4. presents the NOEC and LOEC values obtained to the tested sublethal endpoints with respect to growth and reproduction parameters.

Table II.4. – NOECs and LOECs determined for sublethal endpoints
Bx = Brood number ; IGR = Intrinsic Growth Rate ; DGR = Daily Growth Rate ; a) not determined

Endpoint	**NOEC (µg/L)**		**LOEC (µg/L)**	
	D. magna	*D. longispina*	*D. magna*	*D. longispina*
Total Neonates	0.0844	> 0.1002	0.1266	a)
B1 Neonates	0.1266	> 0.1002	0.1898	a)
B2 Neonates	0.1266	> 0.1002	0.1898	a)
B3 Neonates	0.0844	> 0.1002	0.1266	a)
IGR	0.1266	0.0759	0.1898	0.1002
DGR	a)	> 0.1002	a)	a)

Reproduction

By comparing the cumulative neonate amount in each brood, one has a better overview of the medium-term effect in population growth, caused by concentration increase - Fig II.4..

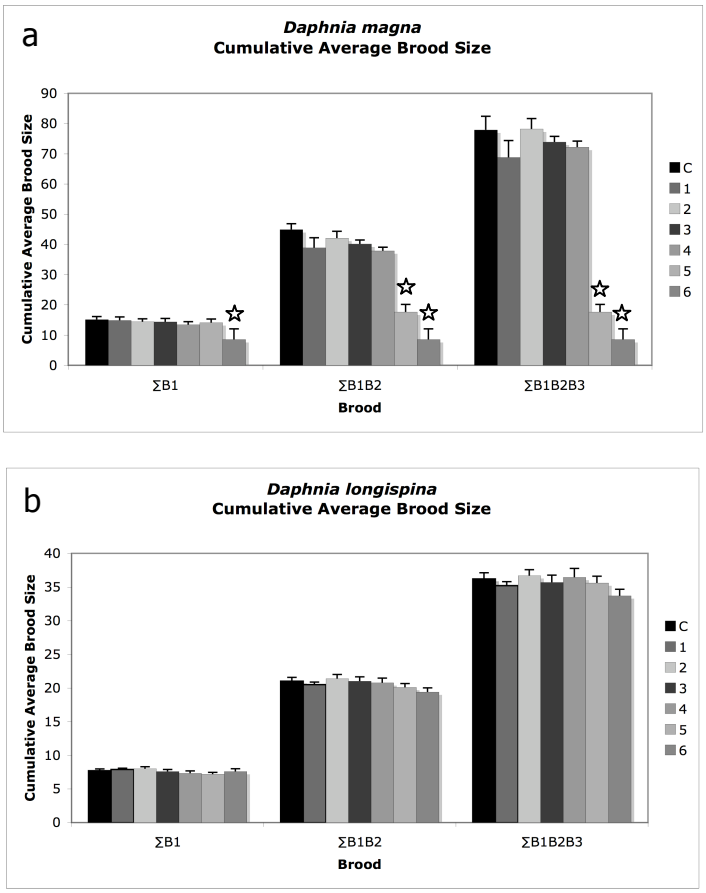

Fig II.4. – Cumulative number of neonates after each brood (B) at different xenobiotic concentrations for *D.magna* (a) and *D. longispina* (b). Error bars represent standard error and ☆ indicates a significant difference from control values (P<0.05).

The tested xenobiotic significantly affected the fecundity (number of neonates per female) of *D. magna* ($F_{[6,53]\ \sum B1}$ = 1.816, P<0.05; $F_{[6,53]\ \sum B1B2}$ = 27.518, P<0.05; $F_{[6,53]\ \sum B1B2B3}$ = 58.271, P<0.05).

For *D. longispina*, no significant differences were found ($F_{[6,62]\ \sum B1}$ = 0.929, P>0.05; $F_{[6,62]\ \sum B1B2}$ = 1.300, P>0.05; $F_{[6,62]\ \sum B1B2B3}$ = 1.073, P>0.05) in the range

of tested concentrations, nevertheless there is a slight trend for a decrease in fecundity with concentration increase (except in B1).

The average age of females at each brood is represented for both species in Fig II.5..

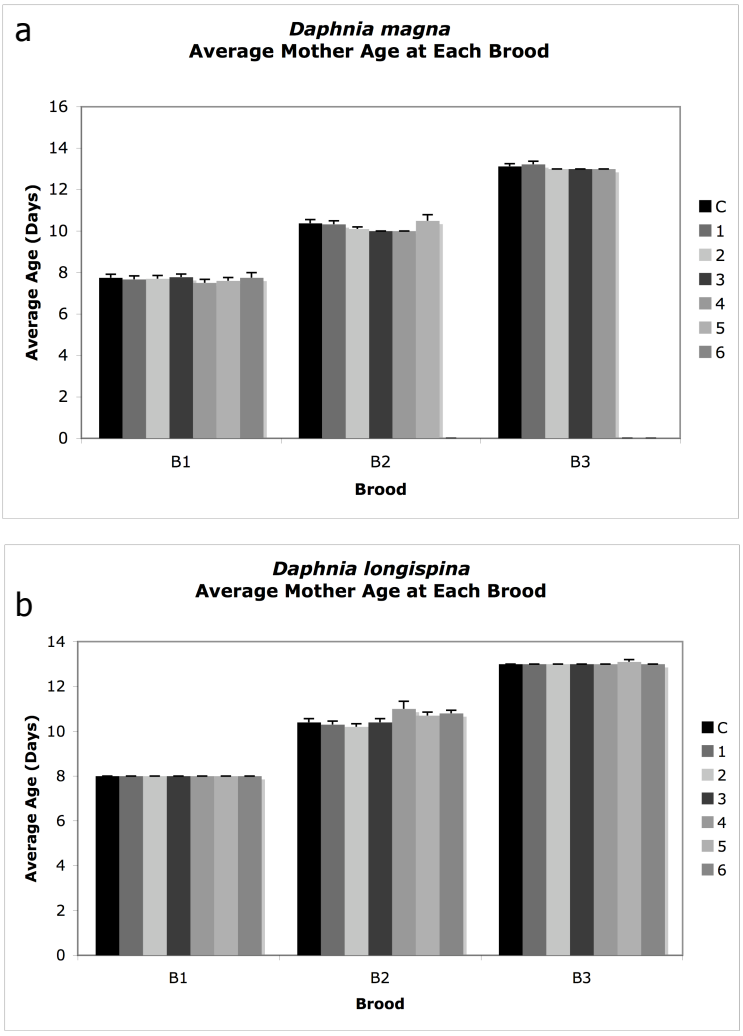

Fig II.5. – Average mother age at each brood (B) at different xenobiotic concentrations for *D. magna* (a) and *D. longispina* (b). Error bars represent standard error.

No significant effects have been found in the 1st Brood in *Daphnia magna* ($F_{[6,53]}$ = 0.364, P>0.05) and all organisms of *Daphnia longispina* released the brood in the 8th day. Nonetheless, a small difference can be noticed, showing that in *D. magna* (a), most of the individuals that were subjected to the xenobiotic released their 2nd and 3rd broods a little earlier, with exception for the highest

concentration, that was in fact, delayed in B2. In *D. longispina* the 1st and 3rd broods were quite synchronized, but the 2nd brood had some differences (not significant), with the higher concentrations (4-6) leading to a slight delay, when the lower concentrations (1-3) eventually lead to a slight advance in the same brood.

One of the most important parameters evaluated in this study was the population's intrinsic growth rate (*r*), which combines both fecundity and survival during the test period, and indicates the population growth along a timeline, where more neonates and early breeding become important factors (Fig II.6.). The *r* shows good information on the effect that a specific concentration can produce, in a population that suffers a long-term exposure to the xenobiotic agent.

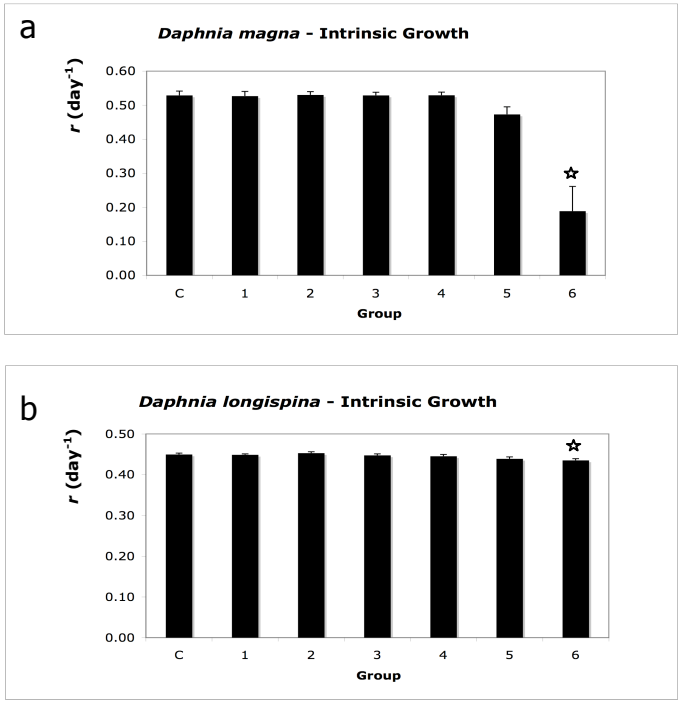

Fig II.6. – Population intrinsic growth rate (*r*) at different xenobiotic concentrations (1-6) and control (C) for *D. magna* (a) and *D. longispina* (b). Error bars represent standard error and ☆ indicates a significant difference from control values ($P<0.05$).

As reported in Fig 6, higher concentrations caused a severe reduction in the population growth of *Daphnia magna*, producing significant effects in both *D.*

magna ($F_{[6,59]}$ = 16.693, P<0.05) and *D. longispina* ($F_{[6,62]}$ = 2.957, P<0.05). Generally, *D. magna* shows higher *r* values in all groups, with exception for the higher concentration used, in which the pesticide effect was greater, thereby significantly decreasing the *r* value.

Somatic Growth

No statistical differences were obtained for the average female size of *D. magna* (Initial: $F_{[6,59]}$ = 1.086, P>0.05; At B1: $F_{[5,50]}$ = 0.715, P>0.05; Final: $F_{[4,41]}$ = 0.344, P>0.05), but for *D. longispina*, significant differences were found for the Final Size, in the 2 highest concentrations (Initial: $F_{[6,62]}$ = 1.490, P>0.05; At B1: $F_{[6,62]}$ = 1.333, P>0.05; Final: $F_{[6,61]}$ = 2.139, P<0.05) (Fig II.7.).

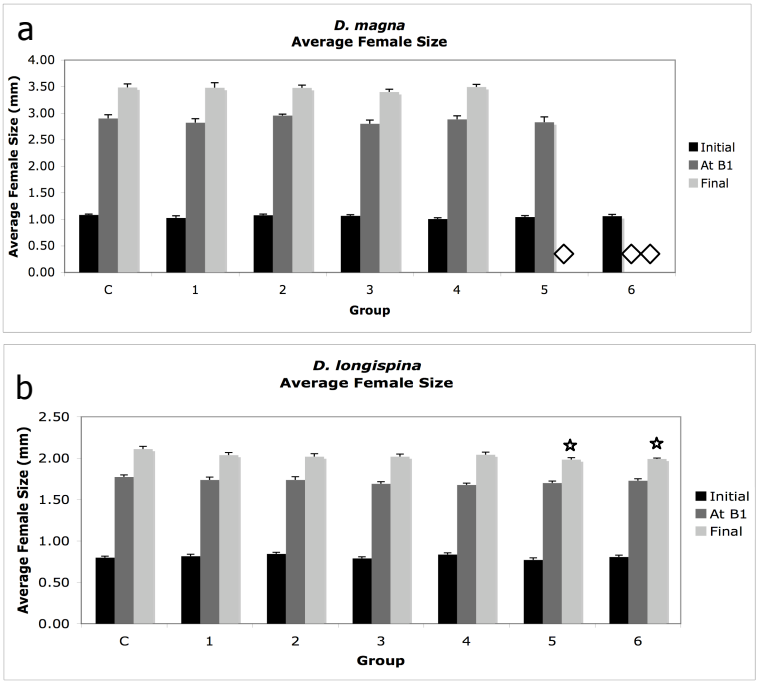

Fig II.7. – Average female size at different xenobiotic concentrations (1-6) and control (C) for *D. magna* (a) and *D. longispina* (b). Error bars represent standard error, ◇ represents unobtainable values, because of moults not being released, and ☆ indicates a significant difference from control

No statistical differences were obtained for the daily growth rate (DGR) of neither *D. magna* ($F_{[4,41]}$ = 1.211, P>0.05) nor *D. longispina* ($F_{[6,61]}$ = 1.227, P>0.05). However, for *D. magna*, all tested concentrations had a higher mean

value than the control. On the contrary, for *D. longispina*, the mean value of all concentrations was lower than the control (Fig II.8.).

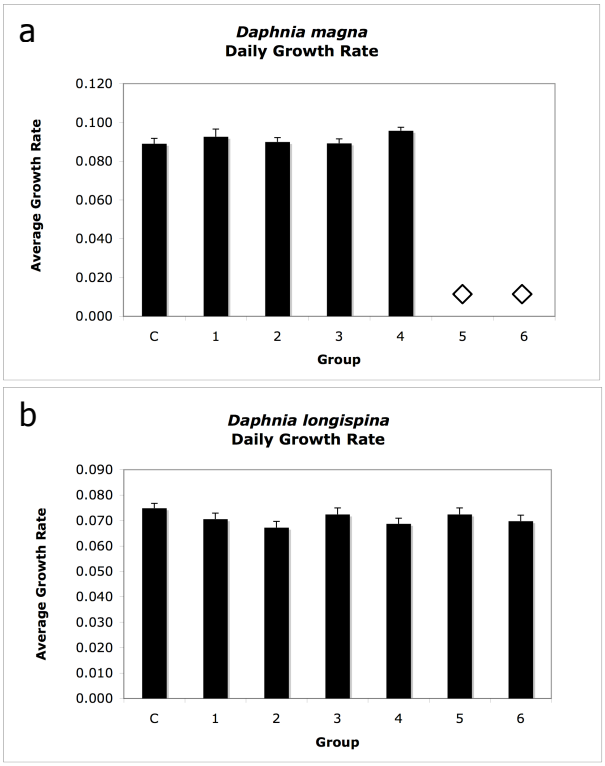

Fig II.8. – Daily growth rate (DGR) at different xenobiotic concentrations (1-6) and control (C) for *D. magna* (a) and *D. longispina* (b). Error bars represent standard error and◇ represents values that were not obtainable because all females died before the 3rd brood

Mortality

Significant differences were found when comparing *D. magna's* mortality from control with the two higher concentrations (according to Fisher's exact test: $P_{(Cx5)}$=0.023; $P_{(Cx6)}$=0.023), corresponding to a LOEC value of 0.12656 µg/L.

Discussion

The results obtained in this study show that Kimlux® induces acute and chronic toxicity to both tested species.

The use of the commercial formulation, instead of the active ingredient alone, has the disadvantage of not being able to evaluate the effects caused by the remaining ingredients. However, the product that is effectively applied in fields is the commercial formulation - in this case Kimlux®. Quinalphos, being the OP active ingredient, and because it is known to cause toxicity to aquatic invertebrates, was the reference substance in this study.

Estimates of *r* and R21 (reproductive output in 21 days) are laborious, time-consuming, and expensive. Therefore, some authors have suggested that the time period should be shortened to 14 days (Tong et al., 1996), 7 to 15 days (Santojanni et al., 1998), single brood (Guilhermino et al., 1999) or even shorter tests (Hanazato, 1998). These suggestions were based on experiments that were designed to compare the results of short-term effects of toxicants, to the standard 21-day test, and with three successive broods being required for a reasonable estimation of *r*, short-term protocols can provide ecologically meaningful data (Trubetskova and Lampert, 2002). In this work, tests were ran until the 3rd brood, observing fecundity, growth, and mortality, and they have revealed significant effects in many cases.

D. longispina, the autochthonous species, showed higher acute sensitivity (EC_{50} = 0.1975 µg/L) to exposures of Quinalphos (about 2.5 times higher) than *D. magna*, the standard species (EC_{50} = 0.5863 µg/L). A possible cause may be the smaller body size of *D. longispina*, which makes for a greater area/volume ratio, therefore more suitable to contamination from water pesticides. Larger juveniles usually are more tolerant to toxic chemicals (Hanazato and Hirokawa, 2004).

In the chronic exposure tests, the fecundity of *D. magna* at B1 was significantly reduced by concentrations ≥0.1257 µg/L (LOEC). The females exposed to concentrations ≥0.0844 µg/L (LOEC) generated almost no offspring on B2, and no offspring at all on B3. The absence of reproduction over higher

concentrations, even prior to death, is in agreement with other works [e.g., Sanchez et al. (1999) with Diazinon; Antunes et al. (2004) with Lindane] that also recorded a decrease of fecundity as response to contamination. In *D. longispina* no significant changes occurred, although, there was a noticeable decrease in fecundity with the increase of concentration, with nearly all tested concentrations producing smaller broods than the control.

The analysis of cumulative values provides a better overview of the tests effects through time, reflecting the importance of persistent effect. In fact, Trubetskova and Lampert (2002) were able to predict the 21 day egg output using data from the first brood. In this case – cumulative fecundity – all *D. magna* females, subjected to concentrations ≥0.08438 µg/L, showed a significant reduction in brood size. In *D. longispina*, none of the tested concentrations did significantly affect the cumulative fecundity. However, the females from the highest concentration did show a noticeable, but not significant decrease, in cumulative fecundity, with the mean difference from control being of -1.5, in B2, and -0.9, in B3.

Regarding the mother age at each brood, no significant changes were found, there was no significant advance or delay in the reproduction day; nonetheless, the second and third broods from the tested concentrations did show a slight advance in reproduction, releasing broods sooner.

The integration of fecundity, age at each brood and mortality (see below), allows the calculation of *r* – the intrinsic growth rate (IGR) of the population. Significant changes were found for both species in the higher concentrations (*D. magna*: $F_{[6,59]} = 16.693$, $P<0.05$; *D. longispina:* $F_{[6,62]} = 2.957$, $P<0.05$). The LOEC value for *r* in *D. magna* was 0.1899 µg/L, and in *D. longispina* was 0.1002 µg/L.

It was in someway expected that a higher concentration would have harsher effects in the population growth in a chronic exposure to an OP (Sanchez et al., 2000), but, in some cases, the population develops adaptations towards the contaminant, through generations, and *r* is able to actually increase (Sanchez et al., 1999; Marques, 2003). Marques et al. (2004) reported *D. longispina* to show an increase in the *r* value as a response to increasing concentrations of

acetylsalicylic acid, which was explained as a life trait strategy of the autochthonous species to the tested xenobiotic or other adverse conditions. This is probably related to the adaptation strategies that the organisms tend to follow, when facing different contamination types. The first brood has a great ecological relevance, because it minimizes the extinction probability of the species in the ecosystems (Stibor and Lampert, 1993). According to Sanchez et al. (1999), the reduction of *r* often results as a consequence of chronic toxicant stress of pesticides on both *Daphnia* species, and only toxicants that cause a substantial decrease in fecundity in the first broods, will cause *r* to decrease significantly. In *D. magna*, the first brood from the highest concentration was significantly affected, thus contributing to a higher decrease in *r*.

Regarding somatic growth, the only significant differences found were in the Final Size of the *D. longispina* females from the 2 highest concentrations ($F_{[6,61]}$ = 2.139, P<0.05). For *D. magna* it is difficult to evaluate this effect, because no moults were released in some cases, and many measurements could not be completed.

The daily growth rate (DGR) values show no significant differences in the tested concentrations. However, for *D. magna*, not enough data could be collected because, again, no moults were released in the 3rd brood from the 2 highest concentrations, and effective growth could not be measured consistently.

An interesting data was the positive correlation existing between the mother's size and the neonate number. In *D. magna*, the mother size at B1 *vs* the B1 Size, had a very significant positive correlation (R=0.342; P<0.01), and for the final mother size (i.e. Size at B3) *vs* total neonate number, a very significant positive correlation was also found (R=0.460; P<0.01). These effects clearly affect the IGR, from which can expect larger females to produce larger broods, and have higher chances of surviving.

Regarding *D. longispina*, a very significant correlation was also found when comparing final mother size with the total neonate number (R=0.411; P<0.01), however, no significance was found when comparing the same parameters in the first brood (B1). Expectedly, the final mother size also had a significant positive

correlation with the *r* of *D. longispina* (R=0.348; P<0.01), meaning that the female's size can have an important role towards population survival. These results come in agreement with other works [Hanazato (1998) with Carbaryl; Santojanni et al. (1998) with Cadmium and Chromium] which showed that the organism's body length is related to body mass, which is related to the amount of resources and their bioavailability.

In general, the chronic exposures to Quinalphos induced a decrease in normal reproduction and growth of both standard (*D. magna*) and autochthonous (*D. longispina*) species. Some studies refer to this difference as a possible toxic effect, by pesticide intake, reducing energy supply, which leads to less growth and consequently less offspring, an in case this happens, population-level effects may occur (Hanazato, 1998; Santojanni et al., 1998; Trubetskova and Lampert, 2002; Marques, 2004; Antunes et al., 2004).

D. longispina did show a higher acute sensitivity to Quinalphos, and maybe its reduced size, and large surface/volume proportion are in someway related to it. However, during chronic exposure the responses were quite similar in both species. If on the one hand, *D. magna* was more tolerant to acute levels of Quinalphos, on the other hand, *D. longispina* had the capability of bearing with the adverse and chronic toxic condition. It is reasonable to conclude that the autochtonous species may have better defense mechanisms to tolerate environmental contaminants.

The concentrations used in this test were very low, but they did present an effect, and if one considers the possibility of a longer exposure to even lower concentrations in the environment, it is likely that cumulative and very discrete effects can be caused to non-target organisms in their natural environments, and worryingly, to the whole ecosystem.

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ACUTE AND CHRONIC EFFECTS OF QUIRLAN® (COMMERCIAL FORMULATION OF CHLORFENVINPHOS) ON THE LIFE-HISTORY PARAMETERS OF *DAPHNIA MAGNA* AND *D. LONGISPINA*.

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Abstract

The uncontrolled use of pesticides can produce drastic effects in the aquatic environment. It is important to inform about the dangerous effects that an abuse in pesticides may cause to many ecosystems. The response of many organisms to these substances is often one of the first tools that can be used to evaluate the effect, and the potential risk of a given xenobiotic to the whole aquatic environment. In this study, the acute and chronic effects of the organophosphorous pesticide Quirlan® (a commercial formulation of Chlorfenvinphos) were observed in growth and in reproduction of *Daphnia magna* (a standard testing species) and *Daphnia longispina* (an autochthonous species). Equal concentrations were used with both species to allow a direct comparison of the effects caused by the xenobiotic. In the acute 48-hour tests, *D. magna* showed less tolerance to this pesticide (48h EC_{50} = 0.687 µg/L) than *D. longispina* (48h EC_{50} = 1.164 µg/L). In the chronic tests, however, this was not so clear, and *D. longispina* had, in fact, higher mortality rates than *D. magna*. There isn't much information on the concentration and effects of Chlorfenvinphos, however, this study revealed its toxicity in some of the tested concentrations. The results show that it is likely that a chronic exposure to even lower concentrations of this pesticide can lead to significant effects in individual and population-level growth and reproduction parameters.

Introduction

It has been reported that approximately 90% of agricultural pesticide applications never reaches its target organisms (Roast et al., 1999). In many aspects, the greatest potential for adverse effects of pesticides is through contamination of the hydrologic system, which supports aquatic life as well as related food chains (Gilliom, 2001). The use of pesticides in agriculture may lead to both surface and ground water contamination by drift, runoff, drainage and leaching (Cerejeira et al., 2003), as well as direct application to treat freshwater crops (Fisher et al., 2000).

The amount of pesticides that will be found in the water will depend on the time period between its application in the field and the rain event, the maximum precipitation and various other soil parameters (Liess et al., 1999). Agricultural soils are the major reservoir of environmental pollutants (e.g., pesticides), and therefore they represent a source from which residues can, often undesirably, be released to the atmosphere and water bodies, and in case these residues become biologically available (i.e., bioavailable), they can contaminate living organisms, specially when used inappropriately by farmers (Goncalves and Alpendurada, 2004). It is the retention, transformation and transport processes as well as their interactions that direct the fate of a pesticide in the soil (Gamón et al., 2003).

According to the 2002 Phytopharmaceutical Product Sales Report by the Portuguese DGPC (Vieira, 2004), 15,501,379 Kg of phytopharmaceutical products (expressed in active substance) were sold in Portugal during the year 2001, from which 254,791 Kg were organophosphorous compounds (OP). The same source revealed that in the following year (2002) there was a 12.6% (1,949,556 Kg) increase in the total of phytopharmaceutical products sold, up to 17,450,935 Kg, while OP sales increased 14.2% (36,128 Kg) up to 290,664 Kg.

Organophosphorous insecticides have been widely used from the 1930s until the present days (Guilhermino et al., 1996; Sogorb and Vilanova, 2002; Ferrari et al., 2004), applied in agricultural fields, forests and other places, in

order to control the quantity of several kinds of pests (Naddy and Klaine, 2001). They became, along with carbamates, the most widely used class of insecticides in the world, replacing the persistent and problematic organochlorine compounds. OPs were initially successful because of their fast degradation and high toxicity (Ferrari et al., 2004), and also because, apparently, they did not accumulate in food chains (Guilhermino et al., 1996). They tend, however, to be less specific (Papp et al., 2004), a fact that can lead to the development of serious problems at the population level, like certain aquatic species (mainly invertebrates) that are affected by these products, while not being its initial target (Barata et al., 2004). This way, OPs can represent a major toxicological hazard when released in the environment (Papp et al., 2004), therefore, they should be carefully selected based on their chemical structure, to ensure a high efficiency against their target pest, while controlling the risk to non-target species by following precautions (PSD, 2005).

A commercial formulation - Quirlan® - with Chlorfenvinphos (molecular structure represented in Fig III.1.) as active ingredient, was used in this study. Chlorfenvinphos is an organophosphorous insecticide, and was one of the most sold pesticides during 2002, in the centre western coast of Portugal. Teixeira et al. (2004) found that 8 people were killed or clinically intoxicated by Chlorfenvinphos, from 2000 to the end of 2002 (only 3 other pesticides intoxicated more people), in the centre of Portugal.

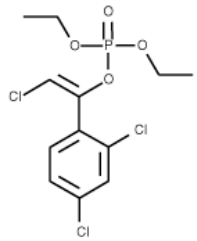

Fig III.1. – Molecular structure of Chlorfenvinphos
[2-chlorophenyl ethenyl diethyl phosphate]

Chlorfenvinphos is considered highly hazardous by the WHO (2002). According to DGPC (2004) this chemical is very dangerous for aquatic organisms, and water contamination should therefore be prevented by not using this substance near watercourses. Nevertheless, this pesticide is one of the most frequently applied in the Portuguese paddies (Pereira et al., 2000), fact that is supported by the high volume of sales (Vieira, 2004). Some of Chlorfenvinphos´ effects have already been studied, and it has been reported that it can cause serious health effects in humans, surprisingly, its presence has even been reported in milk (Kituyi et al., 1997). The bioaccumulation ability of chlorfenvinphos in living tissues represents a potential environmental risk to many different organisms (Serrano et al., 1997).

Daphnia spp. (Cladocera; Branchiopoda; Crustacea) are one of the most widespread zooplanktonic crustaceans in the world, and play a central role in the food webs of pelagic freshwater communities (Vega and Pizarro, 2000). Its high sensitivity to organophosphorous pesticides (Guilhermino et al., 1996; Naddy et al., 2000; Barata et al., 2001), as well as the fact that it often inhabits water bodies near agricultural fields where OP pesticide treatments are more frequently applied, makes it widely used in aquatic risk assessment (Barata et al., 2004).

Daphnia longispina belongs to a complex of many species (i.e., *D. ambigua, D. hyalina, D. galeata, D. cucullata*) and was also used in the present study, because it is an autochthonous species, having been found in several different places in Portugal [e.g., lakes Vela, Braça, Mira and Tapada Grande (Antunes et al., 2003)].

The objective of this study was to examine the acute and chronic effects caused by Chlorfenvinphos while in its commercial formulation (Quirlan®), in both *D. magna* and an autochtonous clone of *D. longispina*. The main emphasis was to evaluate the toxicological responses of both species in order to determine if *D. magna* is representative of local cladocerans.

Material and Methods

STOCK CULTURE OF DAPHNIDS

Parent individuals of *Daphnia magna* Straus clone A [*sensu* Baird et al. (1989)] and *Daphnia longispina* O. F. Müller clone EM7 [*sensu* Antunes et al. (2003)] were both reared in 800 ml of ASTM hard water (ASTM, 1980; EPA, 1989) and fed every two days with *Pseudokirchneriella subcapitata* Korshikov, maintained in our laboratory as described by Stein (1973) in a concentration of 3 x 10^5 cell.ml^{-1} for *D. magna* (OECD, 2000) and 1.5 x 10^5 cell.ml^{-1} for *D. longispina* (Antunes et al., 2003). An organic additive made of *Ascophyllum nodosum* (L.) Le Joli seaweed extract (Baird et al., 1988) was prepared by dilution of a stock solution, and added to the culture medium to a final concentration of 6 ml/L (Soares, 1989). The cultures were maintained in a semi-static system, with a photoperiod of 16^L:8^D and a temperature of 20±1 ºC.

CHEMICALS AND TEST SOLUTIONS

This pesticide was acquired in its commercial formulation, with specifications according to Table IIII.1..

Table III.1. – Pesticide Description

Commercial Name	Active Ingredient	A.I. Concentration
Quirlan®	Chlorfenvinphos	240 g/L

Stock solutions were obtained by dilution of the original products in nanopure water. The fact that the volumes used in each test were quite small, allowed the dilution using ultrapure water instead of the original culture medium (i.e., ASTM Hard Water) thus preventing the occurrence of reactions that could change its properties, prior to the testing period. These solutions were stored at 2-

8ºC in dark glass bottles. No significant changes in pH were noticed in the highest concentrations of this pesticide, which was kept in the range of 6 – 9 pH units.

EXPERIMENTAL DESIGN

ACUTE IMMOBILIZATION TEST

This test was conducted according to OECD's guidelines for the *Daphnia* sp. Acute immobilization test (OECD, 2000), using a single clone of *D. magna* and a single clone of *D. longispina*. Only neonates from the third to the fifth brood with less than 24h were used in these experiments to minimize maternal effects.

Pesticide stock solutions were prepared previous to each test, by dilution in ultrapure water, and kept in the dark and in cold storage. Daphnids were maintained in groups of 5, for a period of 48 hours in glass vessels, with 100 ml of test solutions, with four replicates. The test conditions were similar to those described for the parents´ culture, but neither food nor extract were administrated during the 48-hour period, in order to minimize test variables. Both dissolved oxygen and pH were measured (using the WTW Oxi 330 and the WTW pH 330 meters, respectively) in the beginning and in the end of each test. After the 48-hour period, the number of organisms that remained immotile for approximately 15 seconds after a smooth agitation of the vessel was recorded.

CHRONIC GROWTH AND REPRODUCTION TEST

This test was conducted according to EPA's guidelines for *Ceriodaphnia dubia* Survival and Reproduction Test – Method 1002.0 (EPA, 1989) - adapted for testing with both *D. magna* and *D. longispina*. In these experiments, the same species and clones were used. Again, in order to minimize maternal effects, only third to fifth brood neonates, with less than 24 hours, were used.

Pesticide stock solutions were prepared previously to each test, by dilution in ultrapure water, and kept in the dark, and in a cold storage. For this test, ten

replicates (for the control and for each of six different concentrations) were prepared in 50 ml glass vessels, and filled with the appropriate volumes of ASTM and test pesticide (from stock solution).

Daphnids were individually cultured in each vessel, with the same conditions described for the parents' culture, except that all animals were fed every single day, and were transferred to newly prepared vessels, once every two days. Both oxygen concentration and pH were measured at least once a week to ensure that they were not limiting factors for biological responses. The test was to end after all females released the 3rd brood, or died, to a maximum duration of 15 days.

During the test period, several parameters were observed and recorded. All females were measured three times: in the beginning of the test, after releasing the 1st brood, and in the end of the test. The females' moult is usually lost from its body after all neonates have been released, and for this matter, the length of first exopodite (EL) of the second antennae (Fig III.2.) from each moult, was measured to calculate the female organism's body length in millimeters (equations III.1. and III.2.). This was accomplished because of the allometric relationship that was described between these two variables (Pereira et al., 2004).

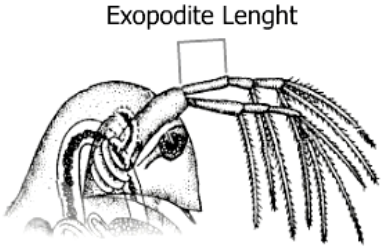

Fig III.2. – Schematic representation of the correct length measurement of the 1st exopodite of the 2nd antenna (EL)

$BL_{D.\ magna} = 10.499 \times EL_{D.\ magna} - 0.329$ (mm) ($r^2 = 0.9392$) (equation III.1.)

$BL_{D.\ longispina} = 10.660 \times EL_{D.\ longispina} - 0.186$ (mm) ($r^2 = 0.9656$) (equation III.2.)

The daily growth rate (DGR) was then calculated (equation III.3.), with BL_f standing for the organism's final body length (mm), BL_i standing for the organism's initial body length (mm) and Δt for the time interval (days) (Burns, 2000).

$$DGR = \frac{\ln(BL_f) - \ln(BL_i)}{\Delta t} \quad (days^{-1}) \qquad \text{(equation III.3.)}$$

The age (days) of each female at each brood, together with the number of offspring, were recorded for each of the 3 broods. Five random first-brood neonates from each vessel were measured for the total body length, this meaning from the top of the head to the base of the spine (Fig. III.3.). All measurements were done using an Olympus SZX9 Stereomicroscope with an ocular micrometer.

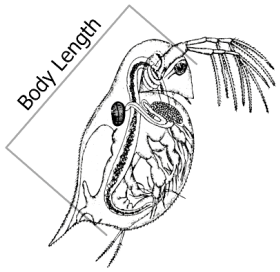

Fig III.3. – Schematic representation of the measurement of the Total Body Length (BL)

The intrinsic rate of population increase (r, day^{-1}) was iteratively calculated using the Euler-Lotka equation (equation III.4.), where x stands for the age class (in days; 0 to n), l_x is the female's survival probability at day x, and m_x represents the fecundity at day x.

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x \qquad \text{(equation III.4.)}$$

All neonates that were released in the same day as the female's death were also counted, as they influence further population growth. Standard deviation for this test was calculated according to the Jackknife technique (Meyer et al., 1986).

DATA ANALISYS

ACUTE IMMOBILIZATION TEST

The number of immobilized animals was plotted against the concentration, to calculate the organism's 48-hour EC_{50} value for the given pesticide at a 95% confidence limit, with the use of Probit Analysis (Finney, 1971).

CHRONIC GROWTH AND REPRODUCTION TEST

The statistical comparison, for each assessed variable, was achieved with the use of one-way ANOVAs, followed by post-hoc Dunnett's tests, to compare results from each treatment and the control (Zar, 1996). In all statistical tests, the level of significance was set at 5%.

The mortality caused by the tested conditions was analyzed using Fisher's Exact Test (EPA, 1989). Both NOEC (No Observed Effect Concentration) and LOEC (Low Observed Effect Concentration) for some of the reproduction and growth parameters were also calculated with the use of one-way ANOVAs, followed by post-hoc Dunnett's tests.

Results

ACUTE IMMOBILIZATION TEST

At the end of the 48-hour test, the EC_{50} determined for *D. magna* (0.6868 µg/L) was about 2 times smaller than the one determined for *D. longispina* (1.2283 µg/L) (Table III.2.).

Table III.2. – 48-hour EC_{50} values, with 95% confidence limits (CL), for *D. magna* and *D. longispina* exposed to Quirlan® (n=20)

D. magna		*D. longispina*	
EC_{50} = 0.6868 µg/L		EC_{50} = 1.2283 µg/L	
Lower CL	Upper CL	Lower CL	Upper CL
0.6428 µg/L	0.7322 µg/L	1.1644 µg/L	1.2964 µg/L

CHRONIC GROWTH AND REPRODUCTION TEST

Table III.3. presents the NOEC and LOEC values obtained to the tested sublethal endpoints with respect to growth and reproduction parameters.

Table III.3. – NOECs and LOECs determined for sublethal endpoints
Bx = Brood number ; IGR = Intrinsic Growth Rate ; DGR = Daily Growth Rate ; a) not determined

Endpoint	**NOEC (µg/L)**		**LOEC (µg/L)**	
	D. magna	*D. longispina*	*D. magna*	*D. longispina*
Total Neonates	0.1013	0.1013	0.1519	0.1519
B1 Neonates	> 0.1519	> 0.1519	a)	a)
B2 Neonates	> 0.1519	> 0.1519	a)	a)
B3 Neonates	> 0.1519	0.0450	a)	0.0675
IGR	0.1013	0.1013	0.1519	0.1519
DGR	0.1013	a)	0.1519	< 0.02

Reproduction

Although no significant difference was found in fecundity (total cumulative number of neonates per female) after the first brood ($F_{[6,61]}$ = 0.668, P>0.05 for *D. magna* and $F_{[6,54]}$ = 2.024, P>0.05 for *D. longispina*), the tested xenobiotic did however significantly affect the fecundity after the 2nd brood on the highest concentrations tested for both *D. magna* ($F_{[6,61]}$ = 7.561, P<0.05) and *D. longispina* ($F_{[6,54]}$ = 3.646, P<0.05), as well as after the 3rd brood on the highest concentrations tested for both *D. magna* ($F_{[6,61]}$ = 12.642, P<0.05) and *D. longispina* ($F_{[6,54]}$ = 4.496, P<0.05) as represented in Fig III.4..

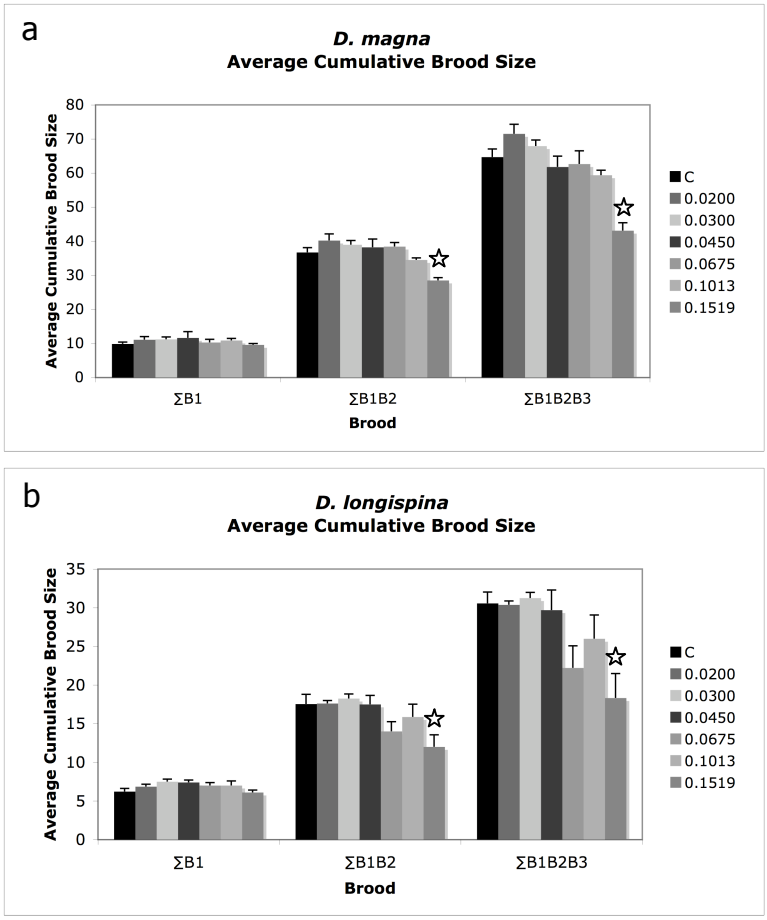

Fig III.4. – Cumulative number of neonates after each brood (B) at different xenobiotic concentrations for *D. magna* (a) and *D. longispina* (b). Error bars represent standard error and ☆ indicates a significant difference from control values (P<0.05).

The average first brood neonate size was always larger in all tested concentrations than in the control, in both species (Fig III.5.). Although no significant differences were found for *D. magna* ($F_{[6,61]}$ = 1.319, P>0.05), for *D. longispina*, significant differences were found in the highest concentration ($F_{[6,55]}$ = 2.441, P<0.05).

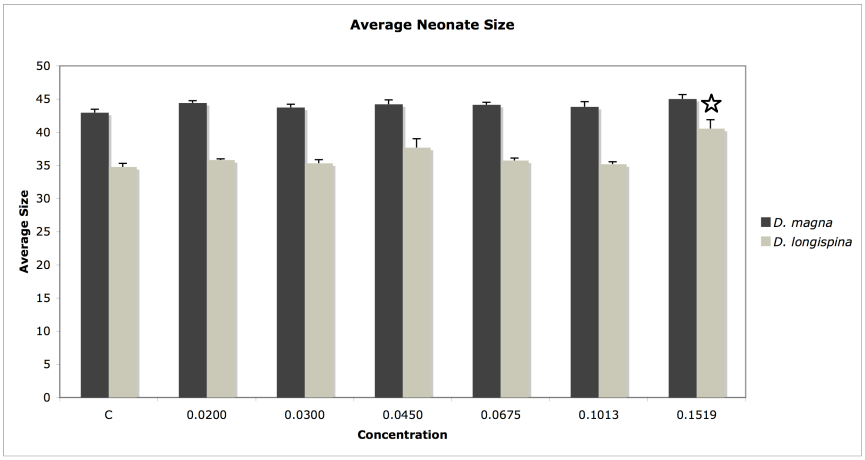

Fig III.5. – Average 1st Brood Neonate Size at different xenobiotic concentrations for *D. magna* and *D. longispina*. Error bars represent standard error and ☆ indicates a significant difference from control values (P<0.05).

The average age of females at each brood is represented, for both species, in Fig III.6, and no significant effects have been found in any of the cases.

No significant differences were found for *D. magna* (1st Brood: $F_{[6,61]}$ = 0.604, P>0.05; 2nd Brood: $F_{[6,61]}$ = 1.512, P>0.05; 3rd Brood: $F_{[6,61]}$ = 0.631, P>0.05), however, in the 2nd Brood there was a slight delay in almost all tested concentrations and also in the 3rd Brood, all tested concentrations had a noticeable delay in the brood release day.

In *D. longispina* the results were also that no significance was found (1st Brood: $F_{[6,54]}$ = 0.856, P>0.05; 2nd Brood: $F_{[6,54]}$ = 0.366, P>0.05; 3rd Brood: $F_{[6,54]}$ = 0.887, P>0.05) and in this case, there are no relevant changes in the mother's age at each of the three observed broods either.

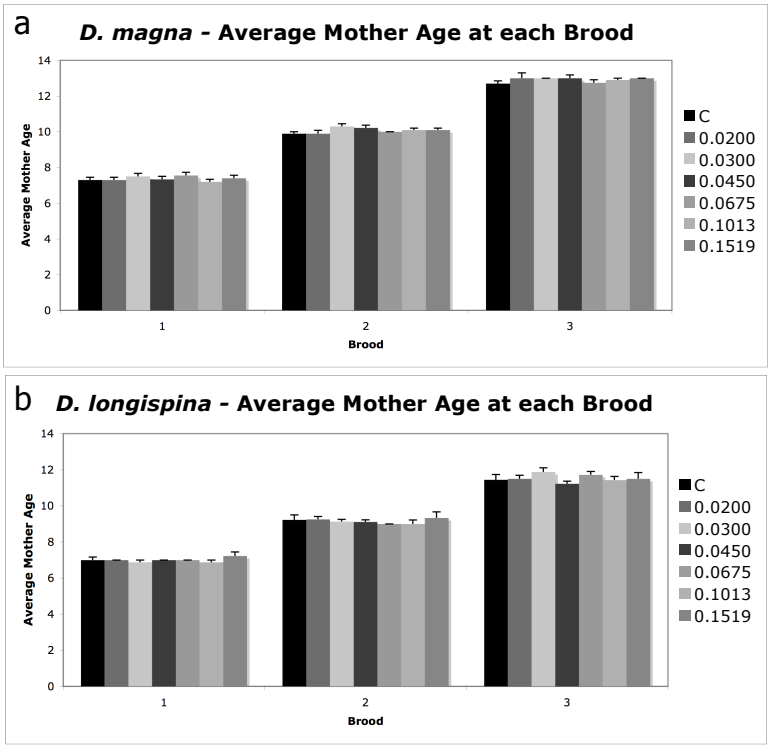

Fig III.6. – Average Mother Age at each Brood at different xenobiotic concentrations for D. *magna* (a) and D. longispina (b). Error bars represent standard error.

The populations' intrinsic growth rate (r) was also calculated for both species. This is a very important parameter because it evaluates the population's growth using fecundity together with survival during the test period, to compute the group's potential success, and growth as a population, along a timeline. Important factors are the number of live neonates as well as early breeding. It is useful to know the effects that the tested xenobiotics may have in the population if organisms are subjected to a long-term exposure to pesticides.

The *r* was significantly decreased in the higher concentrations of pesticide for both *D. magna* ($F_{[6,61]}$ = 3.142, P<0.05) and *D. longispina* ($F_{[6,55]}$ = 2.441, P<0.05). *D. magna* generally shows higher r values than *D. longispina*, but they seem to be affected in the same way (Fig III.7.).

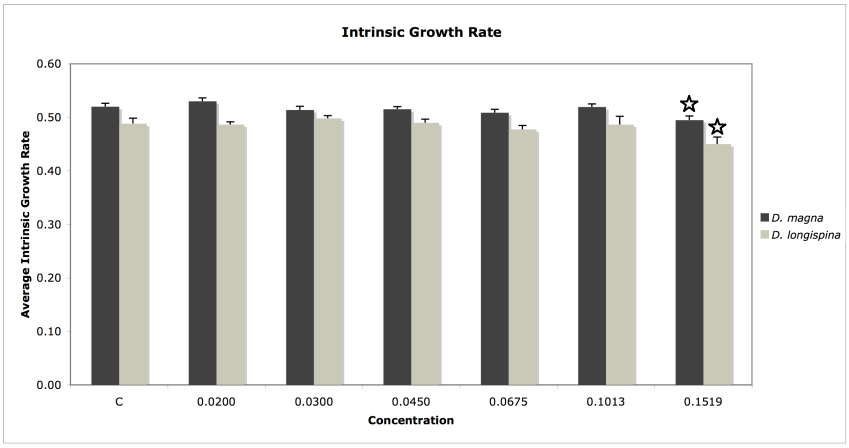

Fig III.7. – Population intrinsic growth rate (r) at different xenobiotic concentrations for *D. magna* and *D. longispina*. Error bars represent standard error, and ☆ indicates a significant difference from control values (P<0.05).

Growth

As represented in Fig III.8., there were considerable and significant effects in growth for both species. In the case of *D. magna*, significant differences were found for the highest concentration only ($F_{[6,58]}$ = 3.540, P<0.05), but in *D. longispina*, the differences were significant ($F_{[6,47]}$ = 10.368, P<0.05) in almost all of the tested concentrations.

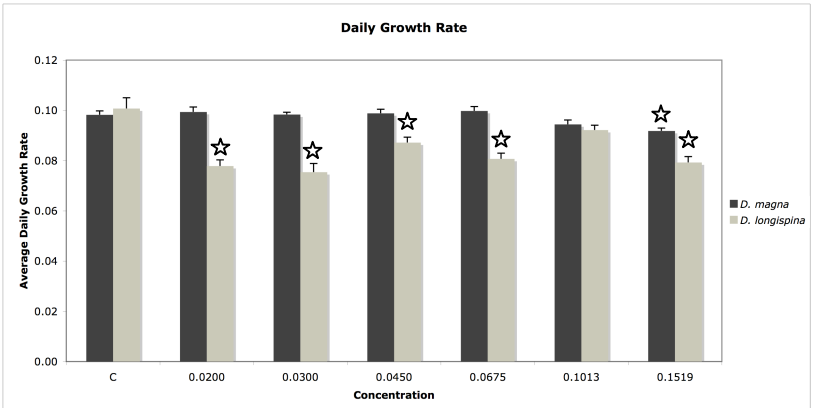

Fig III.8. – Daily growth rate (DGR) at different xenobiotic concentrations for *D. magna* and *D. longispina*. Error bars represent standard error and ☆ indicates a significant difference from control values (P<0.05).

Mortality

No significant differences were found for mortality in the range of tested concentrations.

Discussion

The results obtained in this study show that Quirlan® induces acute and chronic toxicity to both tested species.

The use of the commercial formulation, instead of the active ingredient alone, has the disadvantage of not being able to evaluate the effects caused by the remaining ingredients. However, the product that is effectively applied in fields is the commercial formulation - in this case Quirlan®. Chlorfenvinphos, being the OP active ingredient, and because it is known to cause toxicity to aquatic invertebrates, was the reference substance in this study.

In the acute exposure, *D. magna* (standard species) showed higher sensitivity (EC_{50}=0.6868 µg/L) than the autochthonous species (EC_{50}=1.2283 µg/L). *D. longispina* was almost 2 times more tolerant. It was expected that *D. magna,* would have higher EC_{50} because of the larger body size (Hanazato and Hirokawa, 2004), but that did not occur.

The size of three broods was analyzed by using cumulative amount of neonates in each brood. For ∑B1 (i.e., B1), no significant differences were found in the range of tested concentrations. On the contrary, for ∑B1B2, both species had a significant decrease in fecundity in the higher concentration, corresponding to a LOEC of 0.1519 µg/L. However, the effect in *D. longispina* was more pronounced than in *D. magna.* The total neonate number (∑B1B2B3) also showed a significant decrease in the highest concentration, corresponding to a LOEC of 0.1519 µg/L, and again, the effect in *D. longispina* was more evident.

Higher pesticide concentrations tend to cause higher stress, which has effects in reproduction, and the decrease of reproduction, in higher xenobiotic concentrations, is a response that is frequently described (Sanchez et al., 1999; Sanchez et al., 2000; Marques, 2003; Marques et al., 2004). The aggressive effect in the fecundity of *D. longispina* is aggravated by the fact that this species naturally produces smaller broods, and this can suggest smaller chances of success. In fact, the population dynamics of *Daphnia* in natural freshwater bodies are often controlled by food conditions, predation, temperature and pH. The food

abundance also can vary if there are changes in the *Daphnia* population density. Thus, the vulnerability of these populations to toxic chemicals that is also affected by food abundance may change through time (Hanazato and Hirokawa, 2004).

Another assessed parameter was the average neonate size, and an interesting fact is that the average neonate sizes were always larger for all tested concentrations than for the control. The only significant difference that was found, was for *D. longispina* in the higher concentration tested (LOEC=0.1519), with the neonates being significantly larger in size than the *D. magna* ones. This is probably a response to the xenobiotic contamination, as larger juveniles are likely to have higher tolerance to toxic pesticides and, therefore, to survive longer (Hanazato and Hirokawa, 2004). According to Hanazato (1998), a large juvenile will probably produce a large adult that will almost certainly produce more offspring. Some correlations were found for these parameters. In *D. magna*, the initial mother size showed a positive correlation with the size of B1 neonates ($R=0.298$; $P<0.05$). In *D. longispina*, the initial mother size also presented a very significant positive correlation with the final mother size ($R=0.476$; $P<0.01$). And for both species, the final mother size also had a very significant positive correlation with the total neonates number (*D. magna:* $R=0.655$; $P<0.01$; *D. longispina:* $R=0.391$; $P<0.01$), results that are in agreement with Santojanni et al. (1998) and Sanchez et al. (1999).

The average age of females at each brood for the tested concentrations didn't show significant differences from the control values, meaning that there weren't significant delays nor advances in the brood release days. In *D. magna* one can see a gradual but not significant delay with concentration increase, and in *D. longispina* the tested concentrations also seemed slightly delayed when compared to control.

The population intrinsic growth rate – *r* – is considered one of the most ecologically meaningful parameters [e.g., Stibor and Lampert (1993), Trubetskova and Lampert (2002), Marques et al. (2004)]. It is important, because it estimates the population's long-term success in the environment, by using fecundity data from the organisms together with mortality and the day of each brood. Early

breeding and many neonates are very important to obtain a high *r* value. Chlorfenvinphos induced a significant decrease in *r* in the higher concentrations (LOEC=0.1519 µg/L) for both *D. magna* and *D. longispina*. However, the autochthonous species always had lower *r* values than *D. magna*. One factor that has surely contributed for this effect is the lower fecundity induced by the higher concentrations of the pesticide.

If, in some cases, the population is able to develop adaptations towards the contaminant along generations, and *r* actually increases (Sanchez et al., 1999; Marques et al., 2004), the expected response is a decrease in the *r* value, reflecting the damage done by the toxic agent, that must be able cause a substantial decrease in size of the first broods (Sanchez et al., 2000).

Regarding somatic growth, the daily growth rate of *D. magna* was significantly affected in the highest concentration (LOEC = 0.1519 µg/L; NOEC = 0.1013 µg/L), and noticeable, but not significant differences, were also found for the second highest concentration. For *D. longispina,* DGR was severely affected, with significant differences starting in the lowest concentration tested (LOEC < 0.02 µg/L). This response was not linear, and in the second highest concentration, it wasn't even significant, however, it was significant in all other concentrations, and a great decrease of the DGR was noticed. For the control, *D. longispina* presented a higher DGR value than *D. magna*, which did not occur in any of the tested concentrations, where *D. magna* always presented higher values. A possible cause may be the smaller body size of *D. longispina*, which makes for a greater area/volume ratio, therefore more suitable to contamination from water pesticides. Larger juveniles usually are more tolerant to toxic chemicals (Hanazato and Hirokawa, 2004).

In general, the chronic exposures to chlorfenvinphos induced a decrease in normal reproduction and growth of both *Daphnia magna* and *Daphnia longispina*. This is probably caused by the toxic effect on the organisms, after pesticide intake, reducing energy supply and leading to less growth and less offspring (fecundity) and consequently, population-level effect may occur (Hanazato, 1998; Santojanni et al., 1998; Trubetskova and Lampert, 2002; Marques et al., 2004).

D. magna did show a higher acute sensitivity to chlorfenvinphos, which is a little odd, given the fact that it is larger than *D. longispina*, and therefore, greater resistance would be the expected response. During chronic exposure, *D. magna* was slightly more resistant than *D. longispina*, against the expected response that the autochthonous species would be more resistant to stress situations. However, other variables exist, and they are different species, with different characteristics, which may be responsible for diverse responses found in the two species.

If one considers the possibility of longer exposure periods, at even lower xenobiotic concentrations in the environment, it is reasonable to think that cumulative and sometimes discrete effects may be caused to non-target organisms, and worryingly, to the whole ecosystem.

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General Discussion

This study tested the toxicity of two organophosphorous pesticides (Kimlux® and Quirlan®, commercial products of Quinalphos and Chlorfenvinphos, respectively) in two *Daphnia* species (a standard species *D. magna*, and an autochthonous clone of *D. longispina*). Both pesticides caused acute and chronic toxicity for several different endpoints, in growth and in reproduction of the two cladoceran species.

The two tested pesticides caused similar acute toxicity to *D. magna* presenting close EC_{50} values. However, for *D. longispina*, Quinalphos caused acute toxicity at much lower levels of exposure than Chlorfenvinphos.

Regarding chronic exposure tests, both species were affected in the higher concentrations (~ tenth of microgram per liter), for some of the tested endpoints, and the determined LOEC values were generally higher for *D. magna* than for *D. longispina*, which shows a higher sensitivity from the smaller autochthonous species. This goes in agreement with other studies that also reported higher resistance in larger *Daphnia* species (Lilius et al., 1995), probably because of the smaller surface/volume ratio they present, which grants them higher resistance to toxic substances. However, this is only one of many aspects that influence this organism's behavior and resistance in stress situations, the phenotypic plasticity is a very important parameter that is difficult to evaluate because clonal organisms may show different adaptations to a given situation.

An expected positive correlation between the size of the females and fecundity was also confirmed. Similar effects were found in other studies, which associated the organism's body length with its body mass, and consequently with the amount of available resources (Hanazato, 1998; Santojanni et al., 1998). According to Baird et al. (1990), survival and fecundity are assured by this energy, normally acquired through food intake, and this way, stress situations can affect the energy management processes, and therefore affect growth, reproduction and fecundity of these organisms.

The intrinsic growth rate – *r* – is considered one of the most important and ecologically meaningful parameters (Stibor and Lampert, 1993; Trubetskova and Lampert, 2002; Marques et al., 2004). It is important because it integrates many different parameters (i.e., survival, fecundity, day each brood takes place), to evaluate the probability of success for a given population in a long-term exposure – something like the sustainability of the population. Regarding the *r*, both species were significantly affected by both of the pesticides, in the higher tested concentrations. *D. magna* was highly affected by Quinalphos, that caused severe *r* reduction in the highest tested concentration.

The evaluation of the aforementioned parameters – survival, fecundity and the day each brood takes place – showed some association with each of the tested parameters alone. However, it presented a clearer idea of the sum of the different factors altogether, which is in fact, what happens in the environment. This means that significant effects would probably be noticed in a long-term chronic exposure of these organisms to the tested xenobiotics if they were to be found in the environment, which goes in agreement with the work of Sanchez (2000).

Regarding the assessed somatic growth parameters, Quinalphos caused a significant reduction in the final size of the tested females in the higher concentrations, but no significant differences were obtained for the daily growth Rate (DGR). Chlorfenvinphos, however, had a severe effect in the DGR of *D. longispina* even in very low concentrations (LOEC < 0.02 µg/L). The DGR of *D. magna* was also significantly lower, but with higher acute resistance (LOEC = 0.1519 µg/L).

Mortality was also evaluated in this study, but the only significant effect noticed was with Quinalphos in *D. magna* for the two highest concentrations (LOEC=0.1266 µg/L).

Not much is known about the concentration of these pesticides in the environment, but even if they are found in lower concentrations, the risk of long term chronic exposure is likely to cause discrete cumulative effects to non-target organisms, but also to the entire ecosystem. The position that *Daphnia* spp.

occupies in the food chain makes it very important for the ecosystem, and similar organisms are likely to also be affected by pesticide contamination.

The acute and chronic responses were quite different, which suggests that a long-term exposure analysis may produce more relevant data. Therefore, acute tests may not present, in some cases, enough data for a reliable risk assessment. If a pesticide contamination decreases the chance these populations to survive, other organisms will get affected, and deeper changes may occur in the ecosystem. The fact that toxic substances are constantly affecting non-target organisms in the natural environment may produce severe consequences, and it is therefore important that the legislation is adapted to the present circumstances to avoid the abusive use of pesticides and other contaminants.

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