Universidade de Aveiro Departamento de Biologia
Ano 2009

MARIA PAVLAKI

EFEITOS DA COMBINAÇÃO DE STRESSORES NO CICLO DE VIDA DE *DAPHNIA MAGNA*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica do Doutor Amadeu Mortágua Velho da Maia Soares, professor Catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Susana Loureiro, Investigadora auxiliar do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM).

Για τη γιαγιά Ουρανία και το παππού Μανόλη. Θα έχω πάντα την αγάπη σας και τη σκέψη σας μαζί μου όπου και αν πάω. Σας ευχαριστώ για όσα μου μάθατε...Μου λείπετε!

o júri

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palavras-chave ***Daphnia magna*****, misturas, stressores químicos, stressor natural**

resumo

Os diversos compartimentos naturais estão constantemente expostos a vários tipos de contaminantes, bem como a misturas destes compostos, oriundos da actividade humana. Em ecossistemas aquáticos, vários stressores podem actuar em conjunto e causar danos aos organismos. O aumento do uso de pesticidas para fins agrícolas causa demanda a avaliação do risco inerente que estes compostos representam ao atingir o ambiente aquático. A lixiviação dos pesticidas está geralmente associada com o transporte de matéria orgânica e consequente diminuição da concentração de oxigénio em sistemas aquáticos. Os metais pesados podem ser introduzidos no ambiente através da actividade humana, como a actividade mineira. Portanto, a poluição dos ecossistemas é geralmente caracterizada por uma mistura de compostos químicos, que na maioria das vezes, estão presentes em combinação com stressores naturais Neste estudo, três tipos de stressores químicos, dois insecticidas (imidacloprid e thiacloprid), um metal pesado, níquel, e um tipo de stressor natural, representado por diferentes níveis de alimento foram testados com o objectivo de avaliar o impacto de cada stressor em isolado, bem como as suas misturas e combinações para um organismo não-alvo, *Daphnia magna*, através da aplicação do modelos teóricos Adição de concentração (AC) e Acção independente (AI), bem como seus possíveis desvios para sinergismo (causando efeito mais severo) ou antagonismo (efeito menos severo), dependentes da dose aplicada, ou dependência do rácio entre as doses aplicadas para cada item da mistura e/ou combinação. Os efeitos crónicos dos compostos isolados bem como os das misturas foram obtidos pela exposição de *Daphnia magna* a uma gama de concentrações por 21 dias. Os parâmetros analisados foram a produção de juvenis e o crescimento. Os resultados das exposições isoladas de *Daphnia magna* aos compostos químicos mostraram decréscimo na produção de juvenis e no crescimento do organismo. O mesmo padrão foi observado quando os organismos foram expostos a níveis baixos de alimento, enquanto a níveis elevados, a produção de juvenis e o crescimento foram estimulados. Os resultados da exposição à mistura de imidacloprid e thiacloprid mostraram um desvio do modelo AC, com uma dependência das doses aplicadas, sendo observado sinergismo a doses baixas e antagonismo a doses altas de ambos os compostos. Para a mistura de imidacloprid e níquel, nenhum desvio foi obtido, e a mistura foi ajustada ao modelo AI. A resposta na produção de juvenis para a combinação de imidaclopid ou níquel com níveis baixos de alimento não demonstrou desvio do modelo de acção independente. Para a análise da combinação entre imidacloprid ou níquel com níveis altos de alimento não foi possível aplicar os modelos teóricos, e os efeitos tóxicos dos químicos para os organismos foram avaliados para cada nível de alimento maior que o controlo. Neste estudo, é demonstrado que o impacto dos compostos químicos encontrados no ambiente, em misturas ou em combinação com stressores naturais será diferente do impacto induzido por estes compostos em isolado. O presente estudo mostra a necessidade da avaliação dos efeitos das exposições a misturas de químicos e combinações com stressores naturais encontrados no ambiente, e não somente a avaliação dos compostos em isolado.

keywords

***Daphnia magna*, mixtures, chemical stressors, natural stressor**

abstract

The environment is being constantly exposed to various types of contaminants as well as their mixtures mainly due to human activities. In aquatic ecosystems several stressors may act together and affect the life traits of organisms. The increasing use of pesticides for agricultural purposes will require the assessment of the inherent risk when they arrive in marine or freshwater ecosystems. Pesticide runoffs are usually associated with high inputs of organic matter and depletion of oxygen in aquatic systems. Heavy metals can be introduced into the environment due to human activities such as mining processes. Therefore, polluted ecosystems are characterized by an amalgam of chemical compounds, most of the times in combination with natural stressors. In this study, three different chemical stressors, two neonicotinoid insecticides, imidacloprid and thiacloprid, a heavy metal, nickel and an environmental stressor, food level with low and high concentrations, were tested in order to assess the impact of their single toxic effects as well as their mixtures and combinations with a natural stressor on a non-target organism, *Daphnia magna*, with the use of theoretical models, Concentration Addition and Independent Action as well as possible deviations from them, like synergism, (causing a more severe effect) or antagonism (less severe effect), effects dependent from “dose level” (different deviations at high and low concentrations) or those dependent from “dose ratio” (deviations differ from mixture composition). Chronic effects of the single stressors as well as their combinations were assessed by exposing *Daphnia magna* to a range of concentrations for 21 days. The parameters analyzed were offspring production and body length of *Daphnia magna*. Results from single exposure of *Daphnia magna* to the chemical compounds showed a decrease in offspring production and in the body length of the organism. The same was observed when *D. magna* was exposed to low levels of food while in increased levels of food the offspring production and body length increased. Mixture exposure of imidacloprid and thiacloprid for offspring production showed a deviation from the CA model to dose level dependency indication synergism at low does level and antagonism at high does level of the chemicals. For the mixture of imidacloprid and nickel no deviation was obtained and the mixture fitted the IA model. Offspring production from the combinations of imidacloprid or nickel with low food levels showed no deviation from the IA model. For the combinations of imidacloprid or nickel with high food levels it was not possible to use the theoretical models and the toxic effects of the chemicals to the organism were assessed for each food level higher than the control. In this study, it is presented that the impact of chemicals found in the environment in mixtures between them or in combinations with environmental stressors will be different to the impact the single stressors induce. This study shows the need for evaluation of the exposure effects of mixtures of chemicals and combinations with natural stressors found on the environment and not only the single exposure effects.

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Chapter III – Figure 16. Concentration - response data from 21 days exposure of nickel and low food level showing no deviation to the IA model (2D Isobolic Surface). Control value for food level is $3x10^5$ cells/ml. Concentrations reported as nominal values.

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Chapter I

Introduction

The aquatic environment is considered to be the “ultimate stop” for chemicals of natural source or man-made nature (Sumpter 1998). Several studies have been conducted throughout the years as a need to foresee and comprehend the results of those chemicals in the environment, acting alone, interacting one with the other or even interacting with several natural stressors, like different levels of oxygen, food or temperature that may change their toxicity towards organisms.

There are several ways a chemical can enter in the environment. It can be released as a contaminant or by-product of a manufacturing activity, as a result of the use of a product containing the chemical or from the use of the chemical by an industrial or an agricultural process.

Several chemicals can be found in the aquatic environment: veterinary pharmaceuticals, such as antibiotics, (Kümmerer 2009), pesticides (Klöppel et al. 1997; Olette et al. 2008), like as imidacloprid (Maxim and Sluijs 2007), thiacloprid (Beketov and Liess 2008; Beketov et al. 2008) and diazinon (Osterauer and Köhler 2008) or metals, like zinc, cadmium (Amr et al. 1997; De Schamphelaere et al. 2004; Martins et al. 2004; Faria et al. 2008; Komjarova and Blust 2008) or nickel (Enserink et al. 1991; Deleebeeck et al. 2007; Deleebeeck et al. 2008; Komjarova and Blust 2008; Evens et al. 2009; Kozlova et al. 2009), among other.

Pesticides

In recent years, the use of pesticides in agriculture has been increased in order to control, prevent, destroy, repel or mitigate pests harming crops.

The Food and Agriculture Organization of the United Nations defines pesticide as:

any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies. The term includes substances intended for use as a plant growth regulator, defoliant, desiccant or agent for thinning fruit or preventing the premature fall of fruit, and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport (Food and Agriculture Organization of the United Nations 1985).

Types of pesticides

Pesticides are often categorized by the type of pest they control, if they are chemical or derived from a biological source or production method (Environmental Protection Agency 2009). They are catalogued by their targets: an algaecide for the control of algae, a fungicide for the control of fungi, an herbicide for the control of weeds, an insecticide for the control of insects, a rodenticide for the control of rodents etc.

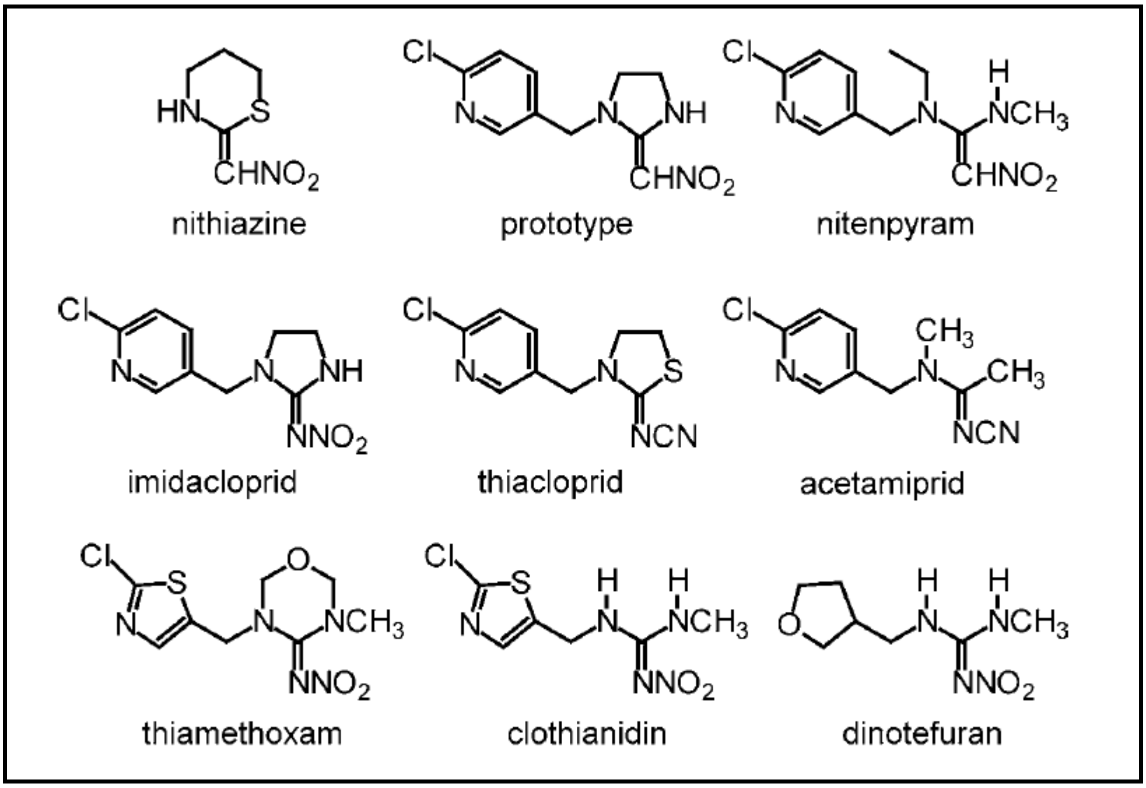

Fig.1. Example of the structure of seven neonicotinoids insecticides used worldwide (Tomizawa and Casida 2005).

Quantities and distribution

EPA (2009) reports that over a billion of pesticide products are used only in the United States per year and according to a statistical study, (Eurostat Statisical Books 2007), a steadily and slight increase in the use of pesticides in 15 countries of the European Union were observed from 1992 till 1999 while after the year 1999 and until 2003, a slight decrease was reported in the 15 countries of European Union. But when 10 new Member States were included in the statistics, it was observed a slight increase in the use of pesticides (Fig. 1, Fig.2 and Fig.3).

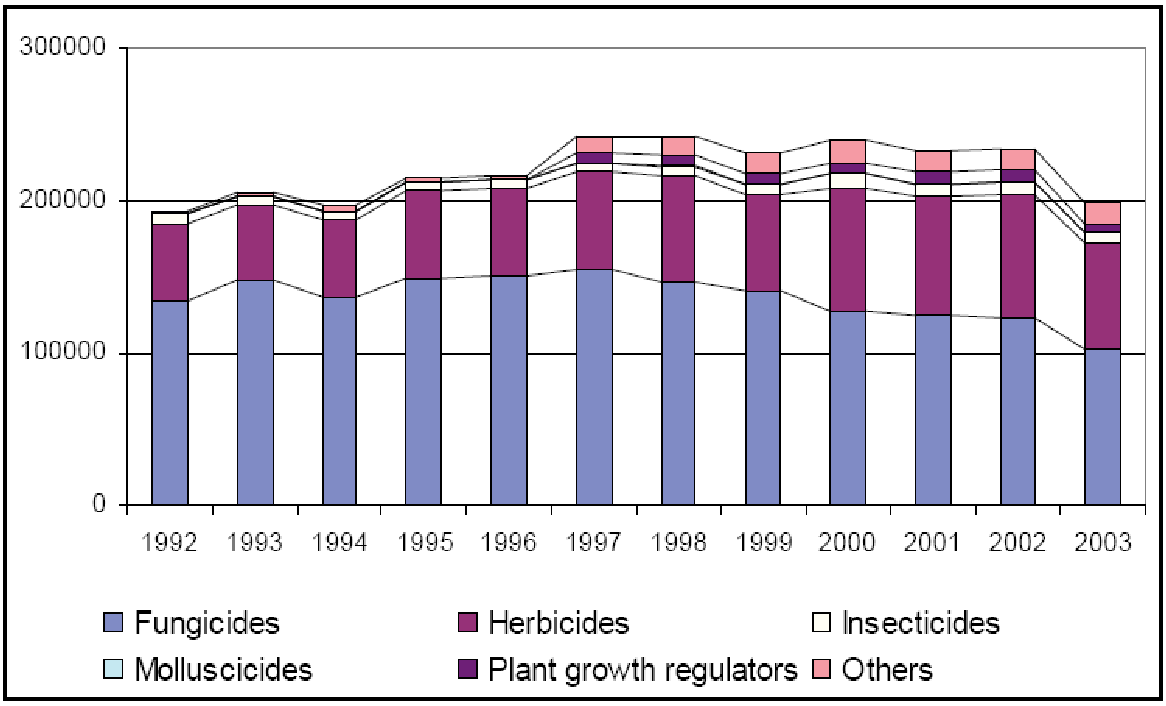

Fig.2. Use and Composition of pesticides used in 15 Member States of the EU from 1992 to 2003 (in tonnes of Active Substance) (adapted by Eurostat Statistical Books, 2007).

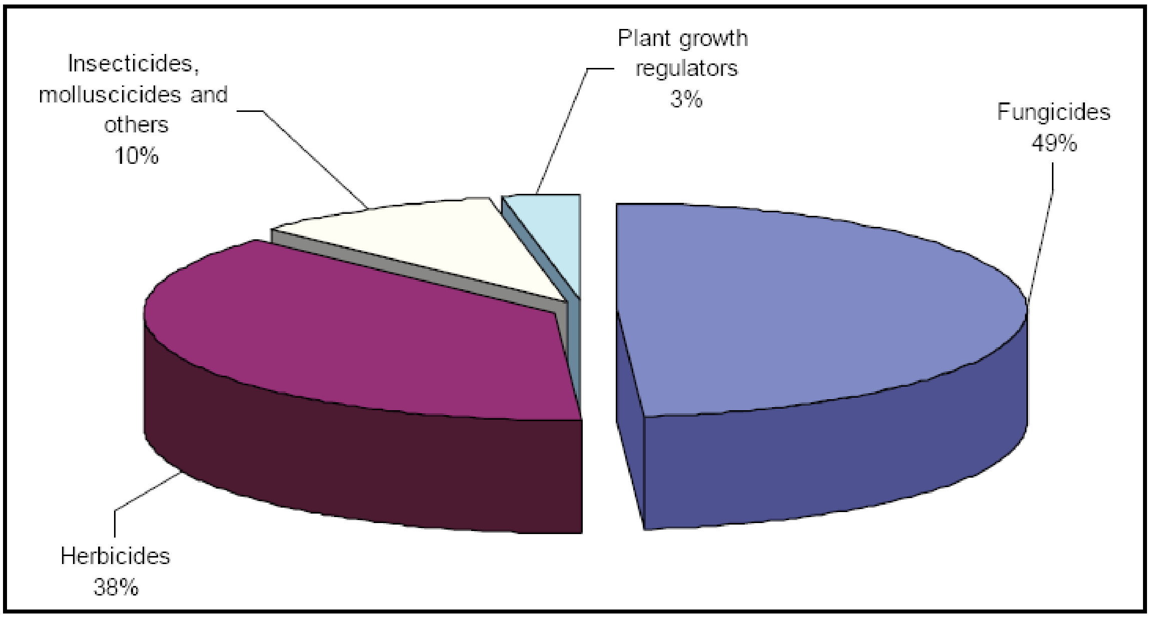

Fig.3. Composition of pesticides used in 25 Member States of the EU from 1999 to 2003 (in tonnes of Active Substance) (adapted by Eurostat Statistical Books, 2007).

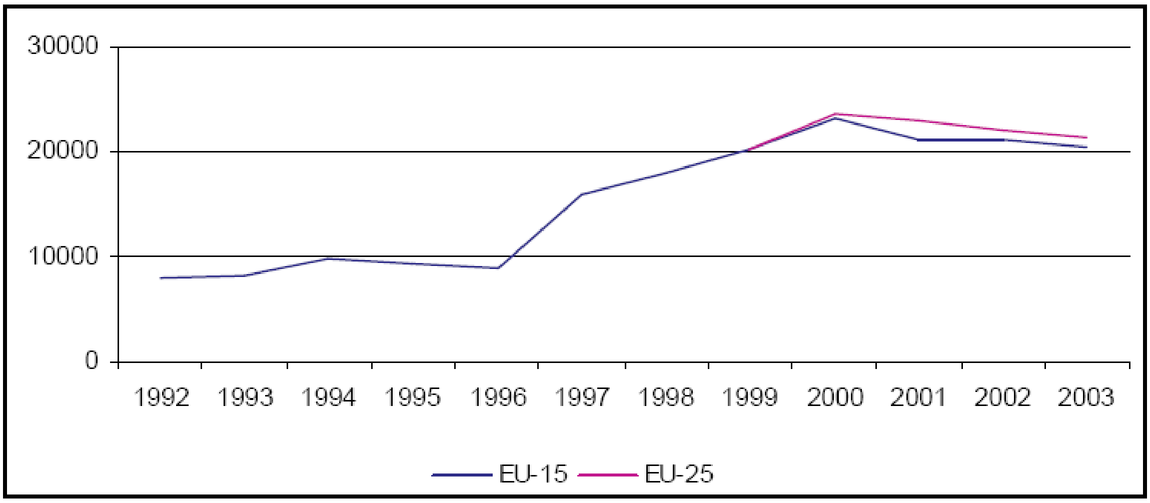

Fig.4. Use and Composition of pesticides used in 15 Member States of the EU from 1992 to 2003 and 25 Member States of the EU from 1999 to 2003 (in tonnes of Active Substance) (adapted by Eurostat Statistical Books, 2007).

Pesticides in the aquatic environment

Pesticides are considered to be one of the main water pollutants and the main routes of entry of pesticides in the aquatic environment can be a) from runoffs of agriculture practices, b) through aerial spray drift, c) with volatilization and subsequent atmospheric deposition and d) through uptake by biota and subsequent movement in the food web (Maas et al. 1984; Rand 1995)

The ecological effects or toxicity of a pesticide and its degradation products (metabolites) to various terrestrial and aquatic animals and plants and the chemical fate and transport of a pesticide in soil, air, and water resources must be assessed and their environmental fate can be affected by different environmental conditions, like the water body characteristics or the climatic conditions.

Regulation

Countries share different regulations to pesticides. For instance, the Environmental Protection Agency registers and licenses pesticides for use in the United States of America. Within the European Union, and according to each Member State, different regulations occur. In the UK, the body that regulates the pesticides for agriculture is the United Kingdom Pesticides Safety Directorate, while the Pesticides Assessment Unit of the Health & Safety Directorate is the body responsible for the regulation of non-agricultural pesticides and biocides in the UK. In Europe, a new EU pesticides legislation was approved by the European Parliament Council that increases the availability of pesticides among the Member States, but at the same time bans the use of certain dangerous chemicals in these products such as the ones that are *carcinogenic, mutagenic or toxic to reproduction, those which are endocrine-disrupting, and those which are persistent, bioaccumulative and toxic (PBT) or very persistent and very bioaccumulative (vPvB)* (European Parliament 2009). Due to the variety of pesticide regulations and in order to deal with no uniformity and heterogeneity among countries, the Food and Agricultural Organization of the United Nations (FAO) has adopted in 1985 an International Code of Conduct on the Distribution and Use of Pesticides, with the objective *to establish voluntary standards of conduct for all public and private entities engaged in or associated with the distribution and use of pesticides, particularly where there is inadequate or no national legislation to regulate pesticides* (Food and Agriculture Organization of the United Nations 1985).

Heavy Metals

A metal is a substance defined chemically as *elements which conduct electricity, have a metallic luster, are malleable and ductile, form cations, and have basic oxides* (Atkins and Jones 1997). According to an IUPAC report the term heavy metal refers to a group of metals and semimetals that are associated with highly toxicity and ecotoxicity and this kind of terminology is considered to be *meaningless and misleading* (Duffus 2002).

Metals in general are naturally occurring and persistent in the environment. They can be essential or non essential to organisms, are readily dissolved and transported by water, taken up by aquatic organisms and bioaccumulated in the fat tissues and their toxicity towards the organisms can be highly influenced by biological, like micro - organisms or geochemical factors, such as presence of organic matter or types of sediment, that influence metal availability (DeForest et al. 2007; Ferreira et al. 2008b).

Quantities and distribution

Earth's composition is mainly dominated by metals, such as iron, magnesium, nickel, aluminum while the crust contains low quantity of metals, like iron, aluminum or magnesium as the main ingredient is oxygen followed by silicon.

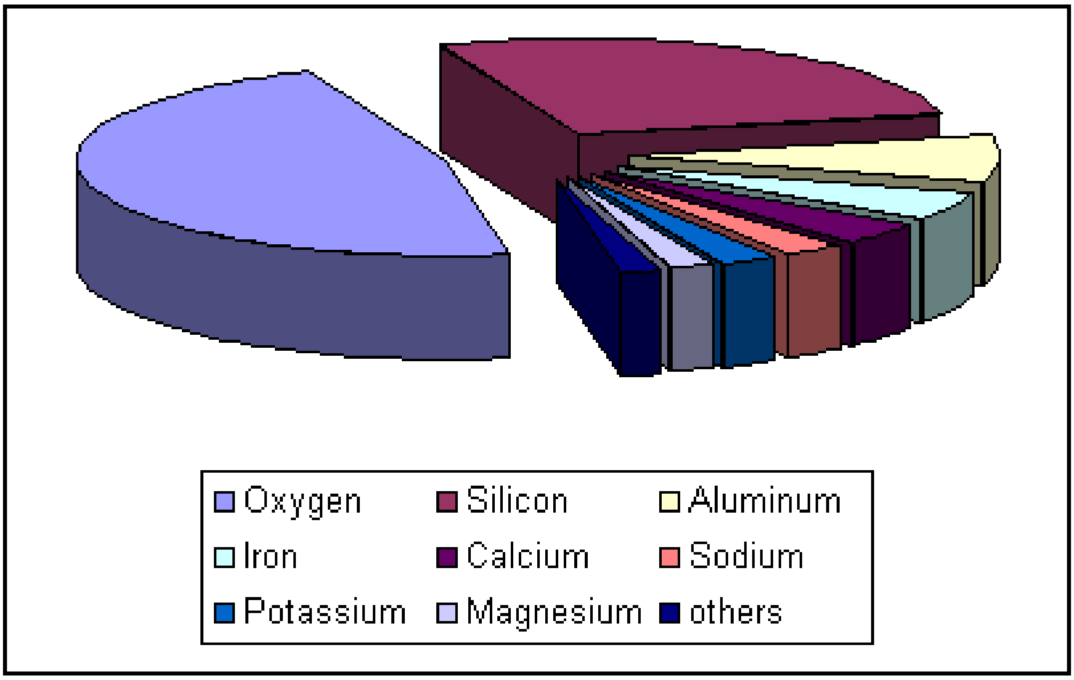

Fig.5 Chart of the composition of the Earth's crust. Source from http://geography.sierra.cc.ca.us/booth/Physical/chp13_earth.aspx.

Europe consumes 20-35% of global metal supply while its global production of metal reaches only a few percent (Nurmi et al. 2008). However, the mining industry in Europe is declining and the production has been suppressed while in the last decades many mining sites have closed throughout most European countries (Kaliampakos et al. 2003). At the same time, global consumption of metals is growing rapidly, approximately 197.000 tones (t) of nickel are currently produced per year in Canada, most of which is sold abroad while in China the production and demand of zinc is high (Fig.7), mainly due to enormous industrial growth and infrastructure building in China and other developing countries (Nurmi et al. 2008).

According to Nurmi (2008) Europe has a long mining tradition and has a number of important metallogenic districts including the Fennoscandian Shield, the Poland-Germany Cu belt, the Carpatho-Balkan Polymetallic belts, the Iberian belt and the Irish base metal belts. The deposit data base of the Fennoscandian ore lists over 120 large or potentially large metal deposits in the region, including Fe, Ni, Cr, Cu, Zn, Au and Mo deposits.

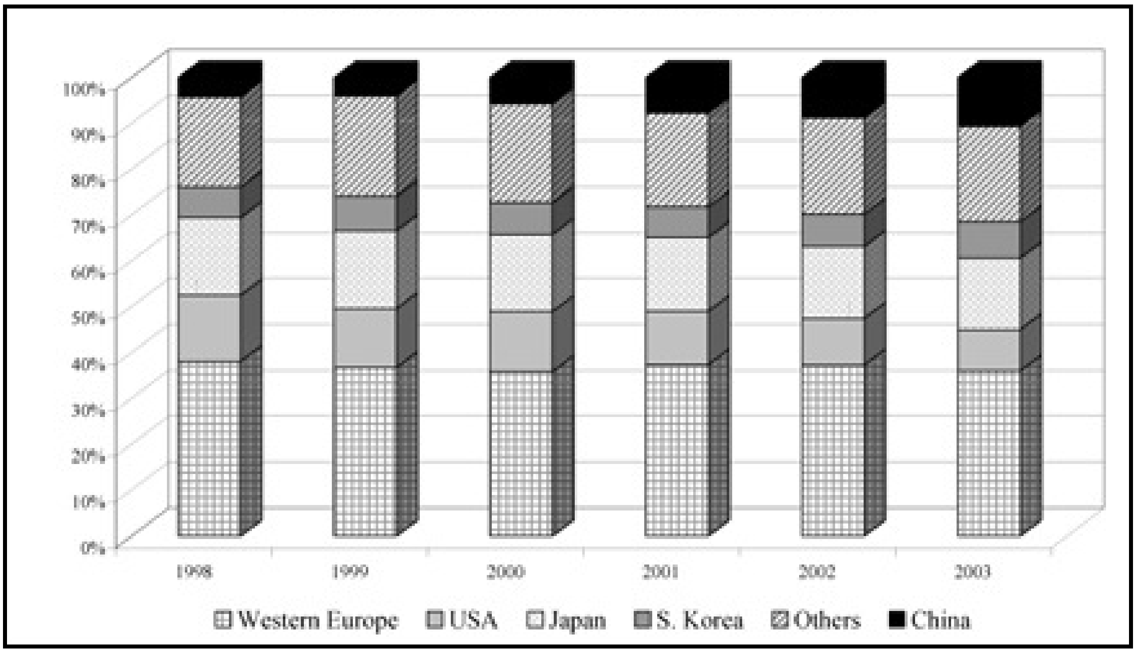

Fig.6 Example of the movement of world's nickel demand. Source from http://content.edgar-online.com/edgar_conv_img/2005/01/07/0001193125-05-002708_G67316019.JPG.

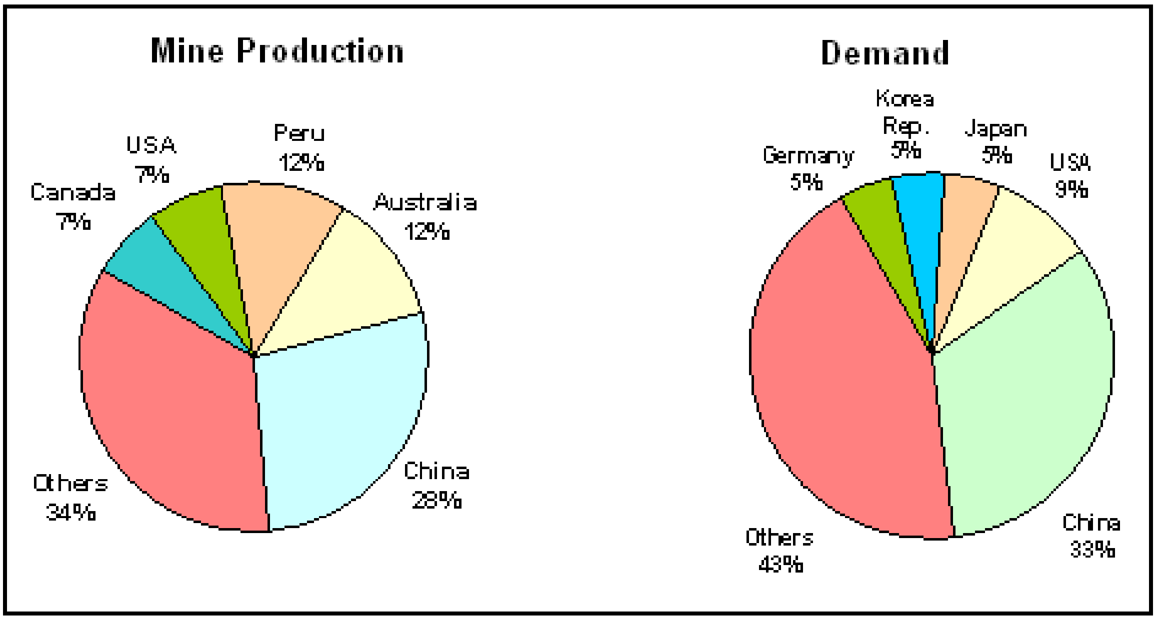

Fig.7 Mine production and demand of Zinc in the world in the year 2008. Source from http://www.ilzsg.org/static/home.aspx.

Metals in the aquatic environment

Heavy metals can enter in the aquatic environment as a result of natural weathering and erosion of geological material (Haynes and Johnson 2000). They can also be released as a result of human activities including mining, smelting, refining, alloy processing, scrap metal reprocessing, other metal operations, fuel combustion, and waste incineration (Kszos et al. 1992; Baptista Neto et al. 2000).

Several studies have detected different levels of metals, such as nickel (Catsiki and Panayotidis 1993; Tsangaris et al. 2007), cadmium (Das and Jana 2003), mercury and several others (Faria et al. 2006), in the environment due to natural or human processes (Barbosa and Hvitved-Jacobsen 1999; Costa and Jesus-Rydin 2001).

Regulation

The World Health Organization (WHO) has developed certain guideline values of metals in order to define the water quality that may be safely consumed.

According to EU legislation, Directive 2006/66/EC for heavy metals in batteries and accumulators, batteries containing hazardous substances, like heavy metals, their marketing will be regulated or prohibited in order to assure the environmental safety and introduces measures for collection and recycling batteries.

In the United States and according to the Environmental Protection Agency there are several metals that have not yet been regulated for drinking waters. For instance, inorganic chemicals such as metalloid elements like nickel, aluminium, molybdenum or boron.

Combined Stressors in the environment

The potential impact of combined effects from co-occurring stressors in the aquatic and terrestrial environments has drawn the attention of scientists and led to numerous studies of mixtures during the past 30 years (Belden et al. 2007).

Several toxicity studies have evaluated the effects of mixtures of two or more stressors to different species following laboratory exposures (Enserink et al. 1991; Jak et al. 1996; Cedergreen et al. 2007; Xie et al. 2007; Ferreira et al. 2008a; Syberg et al. 2008; Loureiro et al. 2009; Martin et al. 2009), and in situ bioassays (Tsangaris et al. 2007; Faria et al. 2008).

Mixture toxicity analysis can be approached in various ways (Greco et al. 1995; Jonker et al. 2004). In order to evaluate the potential of mixtures and predict and assess the possible effect they will cause, reference models have been developed in the beginning of the 20th century. There are two basic conceptual models used for predicting the effect of a mixture from the individual chemicals consisting it, when the composition of the mixture is known, 1) Concentration Addition (CA) (Loewe and Muischnek 1926) and 2)

Independent Action (IA) (Bliss 1939). Concentration addition assumes that substances with a similar mode of action have a common target site in the organism and it is used to predict the effect of those substances will cause (Loewe and Muischnek 1926). Substances with dissimilar mode of action and different target sites, their effects are better predicted by the Independent Action concept (Bliss 1939).

Chemical mixtures may occur in the environment in a big range of concentrations, and do not always follow the response pattern of the two conceptual models, CA and IA, but show more complex deviations patterns such as those causing a more severe (synergism), or less severe (antagonism) effect, those dependent from “dose level” (different deviations at high and low concentrations) or those dependent from “dose ratio” (deviations differ from mixture composition) (Jonker et al. 2005).

Objective

The main goal of this study was to determine the impact of combined stressors to the non-target organism *Daphnia magna*. For that two insecticides, imidacloprid and thiacloprid, a heavy metal, nickel and a natural stressor, food level, were used and tested singly and as binary combinations: imidacloprid – thiacloprid and imidacloprid – nickel, and imidacloprid – food levels and nickel – food levels on a non target organism.

Ecotoxicological tests were used to evaluate the chronic toxicity of each single stressor and their combinations in a non target organism, the cladoceran *Daphnia magna.*

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Chapter II

Effects of binary mixtures on the life traits of *Daphnia magna*

Effects of binary mixtures on the life traits of *Daphnia magna*

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Abstract

The environment is being constantly exposed to various types of contaminants as cocktail mixtures mainly due to human activities. The increasing use of pesticides in agriculture requires the assessment of the inherent risk when they arrive in marine or freshwater ecosystems. Because polluted ecosystems are characterized by an amalgam of chemical compounds, the objective of the present study was to assess the joint effect of the combination of two neonicotinoid insecticides, imidacloprid and thiacloprid, and the effect of the insecticide imidacloprid with nickel chloride on a non – target organism, the cladoceran *Daphnia magna* Straus. Theoretical models have been developed and applied in studies with chemical mixtures, predicting toxicity based on their modes of action: concentration addition and independent joint action models. Still there are cases where deviations are observed (e.g. synergistic or antagonistic behaviours, dose ratio or level dependency). In this study, the effects of the individual compounds and their mixtures were studied in a chronic test where reproduction, survival and body length were evaluated in *D. magna*. Regarding single compound effects, it was observed that according to the EC_{50} values, the most toxic is nickel chloride followed by thiacloprid and imidacloprid. In the chronic mixture exposure of imidacloprid and thiacloprid, a dose level deviation was observed, with synergism at low doses of the chemicals and antagonism at high doses. In the chronic mixture exposure of imidacloprid and nickel, no deviation from the conceptual model was observed. Response patterns for the number of juveniles and daphnids' body length will be discussed considering chemicals mode of action and deviations from conceptual models.

Introduction

Environmental pollution is the contamination of the physical and biological components of the earth/atmosphere system to such an extent that normal environmental processes are adversely affected (Kemp 1998).

The pollution can be categorized into two types I) anthropogenic sources of pollution and II) natural sources of pollution.

I) Anthropogenic sources can be either:

By the object of pollution which in turn can be: a) air pollution, b) water pollution and c) soil pollution.

By the economic source of pollution: a) agricultural pollution, b) industrial pollution, c) transport pollution, that can be categorized as i) car pollution, ii) ship pollution, iii) airplane pollution and d) commercial and domestic sector pollution, or

As other types of pollution such as: a) radioactive pollution, b) chemical pollution, c) invasive species pollution, d) light pollution, e) noise pollution and f) visual pollution.

II) Natural Sources can be either: a) volcanic eruptions, b) dust storms or c) smoke from forest and grass fires (Ignatova 2008).

The majority of the ecotoxicological studies and regulatory risk assessment of chemicals in the aquatic and terrestrial environment focuses mainly on the assessment of the toxicity based on the evaluation of the effects of single compounds in controlled conditions (Barata et al. 2006). However, organisms in the environment are constantly exposed to a mixture of stressors and not only to a single compound or fluctuations on abiotic factors. Anthropogenic chemical contamination is a result of the increasing number of single toxicants entering in the environment, related to industrial, agricultural and urban activities, resulting in complex mixtures of those toxicants (Jonker et al. 2004; De Zwart and Posthuma 2005; Cedergreen et al. 2008; Ferreira et al. 2008).

Imidacloprid (IMI) is at the moment the insecticide with the world's fastest growing sales (Tomizawa and Casida 2005) and is considered as a possible replacement for the widely used organophosphorus pesticide, diazinon, which is subject to phased revocation in many countries (Jemec et al. 2007). The use of imidacloprid has been increased since 1991 (Elbert et al. 1991). It is a relatively new systemic insecticide chemically related to the tobacco toxin, nicotine(Cox 2001), acting as an agonist at nicotinic acetylcholine receptors (nAChRs) and shows selective toxicity for insects among invertebrates (Matsuda et al. 2001), disrupting the nervous system of the insect (Tomizawa and Casida 2005; Fossen 2006) by binding or partial binding to specific areas of the AChR (Anatra-Cordone and Durkin 2005), being considered a neurotoxin, it can be considered as a potential contaminant for surface water and groundwater through runoffs from agricultural areas where it has been used (Gupta et al. 2002; Fossen 2006; Jemec et al. 2007).

Thiacloprid, as imidacloprid, is a neonicotinoid, or chloronicotinyl, insecticide, relatively new and shows high selective toxicity to insects by exhibiting specific activity against the nervous system of the insect (Posthuma et al. 2002; Beketov and Liess 2008). Thiacloprid follows the same path, as imidacloprid, for being considered as a potential contaminant of groundwater and surface water after runoffs or even contaminate the water body through air drifting transfer after being applied by spraying an area. However, as mentioned by Beketov (2008), there is not enough information when it comes to the toxicity of this insecticide to non target freshwater organisms nor the way it may react or its consequences after entering in the aquatic ecosystem.

The toxicity of metals when entering the aquatic environment varies widely, depending on both environmental conditions and the sensitivity of the exposed organisms (Kozlova et al. 2009). They are considered to be one of the most resistant pollutants in waters. Nickel (Ni) is one of the most naturally occurring and abundant elements in the Earth and an anthropogenic contaminant in aquatic and terrestrial environments. Nickel compounds are widely used in modern industry and are sources of environmental contamination from the industrial sewage, through production, processing, and recycling of various Ni products, including stainless steel, electroplating, pigments, and ceramics (Chowdhury et al. 2008; Vandenbrouck et al. 2009) or by atmospheric settle down by precipitation. Ni concentrations usually appear to be below 10 µg/L, around 0.5 to 5 µg/L, in uncontaminated natural waters but may reach up to 0.2 mg/L or in even higher levels in strongly Ni-contaminated sites (Pane et al. 2003; Chowdhury et al. 2008; Kozlova et al. 2009) where values of 2.5 mg/L can be measured (Vandenbrouck et al. 2009). Pane (2003) linked the impacts of *D. magna* in hard water to disruption of Mg^+ balance in the organism. Nickel appears to suppress the reproduction and growth of daphnids when it is found in concentrations higher than 40 ppb and at 80 ppb can come a 100% lethally in *D. magna* after a 21-days of exposure (Munzinger 1990). However, those values depend on different physico-chemical parameters, e.g. water hardness, which plays a significant role on metal's toxicity. Water hardness is considered as a protective mean against the toxicity of the cationic metals (De Schamphelaere and Janssen 2002; Deleebeeck et al. 2007), such as nickel (Hoang et al. 2004; Niyogi and Wood 2004; Deleebeeck et al. 2007; Deleebeeck et al. 2008).

Since a chemical will rarely be found alone in the environment but commonly in combination with others, several studies have been conducted in order to assess in a more realistic way the behaviour of contaminants when they occur in the environment. Studies have conducted in the aquatic (e.g. Backhaus et al. 2003; Syberg et al. 2008) as well as the terrestrial environment (e.g. Drobne et al. 2008; Gomez-Eyles et al. 2009; Loureiro et al. 2009).

In order to observe and assess the toxic effect caused by a number of stressors, two reference concepts are well establishes, the Concentration Addition (CA) (Loewe and Muischnek 1926) and the Independent Action (IA) (Bliss 1939). However, deviations from this two concepts may occur in complex mixtures of chemicals, deviations such as synergism or antagonism, dose level and dose ratio dependency exist (Jonker et al. 2005).

Binary mixtures are usually used aiming to enlighten the way of two different chemicals may be acting when they will be found in the environment, by analyzing the effect of the one on the biological action of the other (Cedergreen et al. 2007) and due to the facility of interpreting effects in contrast to mixtures with more than two chemicals.

The option for using either the Concentration Addition model or the Independent Action model is clearly based on the mode of action of each stressor. If the stressors have similar mode of action then the Concentration Addition model is chosen over the Independent Action while when they have dissimilar mode of action the Independent Action model is preferred and in the case where the mode of action of the stressors is unknown, both models are being used and the one that best fits the data is chosen over the other.

Daphnia magna is widely used as a test organism in order to evaluate the toxicity of several stressors as well as their mixture. The cladocerans are considered to be one of the most sensitive organisms to pesticides and metals (Sanders and Cope 1966; Prabhu and Pierre 1995; Keithly et al. 2004).

The purposes of this work were: (1) to assess the sublethal chronic effects of the two pesticides, imidacloprid and thiacloprid and the heavy metal compound, nickel chloride, and (2) to evaluate the chronic effects of binary mixtures of imidacloprid and thiacloprid and imidacloprid and nickel using the non-target organism, *Daphnia magna*. We hypothesize that the toxicity of the first mixture will be predicted by the Concentration Addition model as both chemicals have similar modes of action and the second one will be predicted by the Independent Action conceptual model as both chemicals show dissimilar modes of action.

Materials and Methods

Test Organism

All experiments were performed using the cladoceran *Daphnia magna* (Fig.8), clone K6 (Antwerp, Belgium) as test organism, which has successfully being cultured at the laboratory from more than a year. Long-term bulk cultures of *D. magna* were maintained in artificial medium, ASTM hard water (ASTM 1998). Glass aquariums (6L capacity) were kept with ASTM hard water, and daphnids were fed with the algae

Pseudokirchinella subcapitata in a ratio of $3x10^5$cells/ml and an organic additive (Marinure seaweed extract, supplied by Glenside Organics Ltd.) (Baird et al. 1989) at a ratio of 6ml per litre of ASTM medium. The cultures were maintained in a controlled temperature chamber at 20ºC at a 16:8 light: dark cycle and culture medium was renewed three times a week. New cultures of daphnids were initiated using the neonates from the third to the fifth brood of the old cultures. According to the OECD procedure, a reference test with potassium dichromate ($K_2Cr_2O_7$) was performed for testing the sensitivity of the daphninds.

Fig.8 *Daphnia magna* Straus, Clone K6

Test Chemicals

Chemical compounds used in this study were the two neonicotinoid insectides imidacloprid (CAS No. 138261-41-3, Bayer, Germany) and thiacloprid (CAS No. 111988-49-9, Bayer, Germany) and the heavy metal nickel (II) chloride hexahydrate (CAS No. 7791-20-0, Merck, Germany).

Exposure medium contamination was controlled by chemical analysis by high-pressure liquid chromatography with UV detector (HPLC – UV) for imidacloprid and thiacloprid and by inductively coupled plasma-mass spectrometry (ICP-MS) for nickel. To control medium contamination for imidacloprid and thiacloprid, chemical analysis was performed to two samples of the lowest and the highest concentrations and two samples of the stock solution, due to the complex experimental design used for mixture experiments. For nickel, the same chemical analysis was performed as described for the insecticides.

Single Compound Chronic Toxicity Tests

Chronic toxicity was assessed for each chemical compound using the OECD 211 guideline, for the *Daphnia magna* reproduction test (OECD 211 Guideline 2008).

Five concentrations for imidacloprid and thiacloprid and six for the nickel, plus a negative control, with 10 replicates of one animal (<24 hours old) each were used for the experimental setup. Tests were maintained in a chamber at 20ºC at a 16:8 light: dark cycle, fed daily with a ratio of $3x10^5$ cells of algae per ml per day and the medium was renewed every other day for 21 days. Reproduction was reported and neonates were removed from the vessels. Each vessel was recorded daily for mortality of the organisms and the mortality of the parent animals did not exceed 20% at the end of the test.

Values for pH, dissolved oxygen and temperature were obtained weekly to check for the validity criteria. At the end of the test the total number of offspring produced per parental animal was calculated and the adult daphnids were measured. Adult daphnids at the end of the test were placed under a stereo – microscope and the body length of each daphnid from the point immediately above the eyespot to the base of the dorsal spine was registered.

The nominal concentrations for imidacloprid ranged from 2mg/l to 10mg/L, for thiacloprid from 0.25mg/L to 2.75mg/L and for nickel from 100µg/L to 350µg/L.

Mixture Toxicity Tests

In the mixture toxicity tests, the number of replicates per treatment was decreased from ten to one to allow the use of more treatments per test, in order to obtain a broader range of the chemical's response to the mixture. In both mixtures, imidacloprid – thiacloprid, and imidacloprid – nickel, a binary combination of different toxic units (TU), where 1 TU was equal to the EC_{50} concentration of each chemical, and a single exposure of the organism to the chemicals were used (Van Gestel and Hensbergen 1997).

Imidacloprid – Thiacloprid Mixture

For the imidacloprid and thiacloprid, the experimental design consisted of the single exposures of the organism to five concentrations of each chemical and to 23 combinations of both chemicals, building a fixed ray design.

The TUs that were used for the setup of the mixture were 0.375 (0.25 + 0.125; 0.125 + 0.25), 0.5 (0.375 + 0.125; 0.25 + 0.25; 0.125 + 0.375), 0.75 (0.5 + 0.25; 0.375 +

0.375; 0.25 + 0.5; 0.125 + 0.625; 0.625 + 0.125), 1 (0.75 + 0.25; 0.5 + 0.5; 0.375 + 0.625; 0.25 + 0.75; 0.125 + 0.875; 0.875 + 0.125; 0.625 + 0.375), 1.5 (0.5 + 1; 1 + 0.5; 0.75 + 0.75), 1.75 (1 + 0.75; 0.75 + 1) and 2 (1 + 1). The nominal concentrations that were used for imidacloprid single exposures ranged from 0.69 mg/L to 5.5 mg/L and for thiacloprid from 0.61 mg/L to 2.45 mg/L.

Imidacloprid – Nickel Mixture

For the imidacloprid and nickel, the experimental design consisted of the single exposures of the organism to five concentrations of each chemical and to 38 combinations of both chemicals, building a fixed ray design.

The TUs that were used for the setup of the mixture were 0.25 (0.125 + 0.125), 0.375 (0.25 + 0.125; 0.125 + 0.25), 0.5 (0.375 + 0.125; 0.25 + 0.25; 0.125 + 0.375), 0.625 (0.125 + 0.5; 0.25 + 0.375; 0.375 + 0.25; 0.5 + 0.125), 0.75 (0.5 + 0.25; 0.375 + 0.375; 0.25 + 0.5; 0.125 + 0.625; 0.625 + 0.125), 1 (0.75 + 0.25; 0.5 + 0.5; 0.375 + 0.625; 0.25 + 0.75; 0.125 + 0.875; 0.875 + 0.125; 0.625 + 0.375), 1.25 (1 + 0.25; 0.875 + 0.375; 0.75 + 0.5; 0.625 + 0.625; 0.5 + 0.75; 0.375 + 0.875; 0.25 + 1), 1.5 (0.5 + 1; 1 + 0.5; 0.75 + 0.75; 0.875 + 0.625; 0.625 + 0.875), 1.75 (1 + 0.75; 0.75 + 1; 0.875 + 0.875)) and 2 (1 + 1). The nominal concentrations that were used for imidacloprid single exposures ranged from 0.69 mg/L to 5.5 mg/L and for nickel from 0.04 mg/L to 0.29 mg/L.

Data Analysis

The EC_{50} values obtained from each single chemical exposure to *D. magna* were calculated by fitting the data to the logistic model, $Y_{(ci)} = Y_{max} / [1 + (c_i / EC_{50})^{\beta}]$, with a $Y_{(ci)}$ response as a function of the maximum response Y_{max}, the exposure concentration c_i, the EC_{50} value and the slope β of the response curve, using the software SigmaPlot vers.10.0. For the NOEC and LOEC values, a One Way Analysis of Variance (ANOVA) was performed and the multiple comparisons Dunnett Method was used to detect differences between the data that followed a normal distribution. For the prediction of mixture toxicity and their modes of action, the conceptual models, Concentration Addition (CA) (Loewe and Muischnek 1926) and Independent Action (IA) (Bliss 1939), were tested. The two models and their deviations (Synergism/Antagonism, Dose Level Dependency, and Dose Ratio Dependency) were compared using the method of maximum likelihood and the best fit was chosen described by Jonker et al. (2005).

Table 1

Interpretation of additional parameters (*a* and *b*) that define the functional form of deviation pattern from concentration addition (CA) and independent action (IA); adapted from Jonker et al 2005.

Deviation Pattern	Parameter *a* (CA and IA)	Parameter *b* (CA)	Parameter *b* (IA)
synergism/antagonism	**a>0:** antagonism		
(S/A)	**a<0:** synergism		
Dose-ratio dependent (DR)	**a>0:** antagonism except for those mixture ratios where negative b value indicate synergism	**$b_i>0$:** antagonism where the toxicity of the mixture is caused mainly by toxicant *i*	
	a<0: synergism except for those mixture ratios where positive b value indicate antagonism	**$b_i<0$:** synergism where the toxicity of the mixture is caused mainly by toxicant *i*	
Dose-level dependent (DL)	**a>0:** antagonism low dose level and synergism high dose level	**$b_{DL}>1$:** change at lower EC50 level	**$b_{DL}>2$:** change at lower EC50 level
		$b_{DL}=1$: change at EC50 level	**$b_{DL}=2$:** change at EC50 level
	a<0: synergism low dose level and antagonism high dose level	**$0<b_{DL}<1$:** change at higher EC50 level	**$1<b_{DL}<2$:** change at higher EC50 level
		$b_{DL}<1$: No change but the magnitude of S/A is DL dependent	**$b_{DL}<1$:** No change but the magnitude of S/A is effect level dependent

Results

Single Compound Chronic Toxicity Tests

According to the OECD 211 guideline (2008), the survival, reproduction and body length of adult *D. magna* were assessed after 21 days of exposure to each chemical. The reproduction was expressed as the cumulative number of offspring per live *D. magna* after exposure and results are shown in Table 2.

Reproduction was suppressed significantly as the concentrations of all three chemicals were increased. The EC_{50} value obtained when daphnids exposed to imidacloprid, thiacloprid and nickel, was 5.5 mg/L, 2.45 mg/L and 289.29 µg/L, respectively.

For imidacloprid and thiacloprid, 10% (at 10 mg/L) and 10%-20% (at 1.75 mg/L, 2.25 mg/L and 2.75 mg/L) of mortality, has occurred, respectively while for nickel, after 15 days of exposure to 350 µg/L (highest concentration) 100% of mortality was observed and a 10% and 20% of mortality occurred to the concentrations 250 µg/L and 300 µg/L, respectively, by the end of the test. No mortality occurred in the control of any of the tests.

Table 2

Toxicity values from 21-days reproduction tests with *D. magna* exposed to imidacloprid, thiacloprid and nickel. NOEC- No Effect Concentration; LOEC- Lowest Observed Effect Concentration; EC_{50}- Effect Concentration that causes 50% of reduction on the endpoint.

Chemicals	**EC_{50} (st. error)**	**NOEC**	**LOEC**	**R^2**
Imidacloprid (mg/L)	5.5 (0.23)	2	4	0.92
Thiacloprid (mg/L)	2.45 (0.09)	0.75	1.25	0.86
Nickel (µg/L)	289.29 (3.08)	200	250	0.86

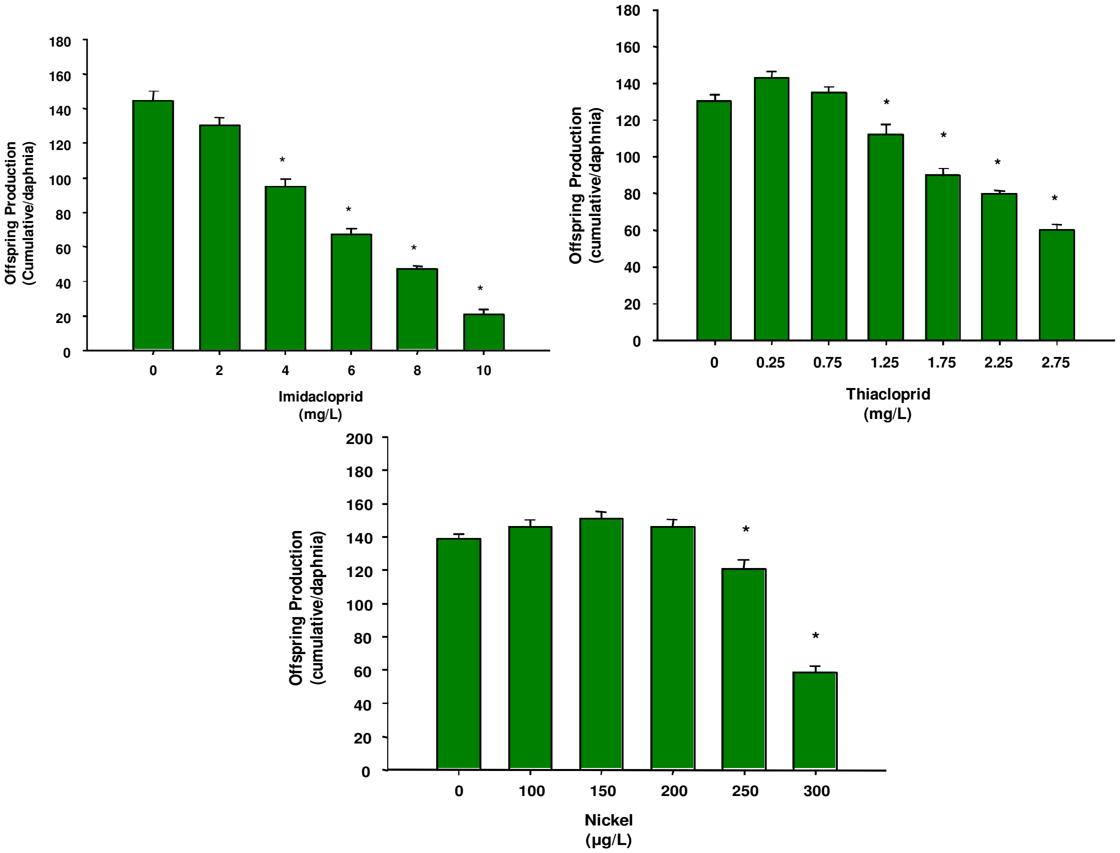

Fig.9 Mean cumulative number of offspring production (and standard error) per live *Daphnia magna* to imidacloprid, thiacloprid and nickel. (*) Denotes significant differences from control (Dunnett Method, P≤0.05).

The body length of adult daphnids was also affected significantly on the tests with imidacloprid and thicaloprid (One Way Anova, $F_{5,52}$= 303.46, P<0.001 and $F_{6,59}$=82.69, P<0.001, respectively), as well as to nickel (One Way Anova, $F_{5,50}$=178.41, P<0.001).

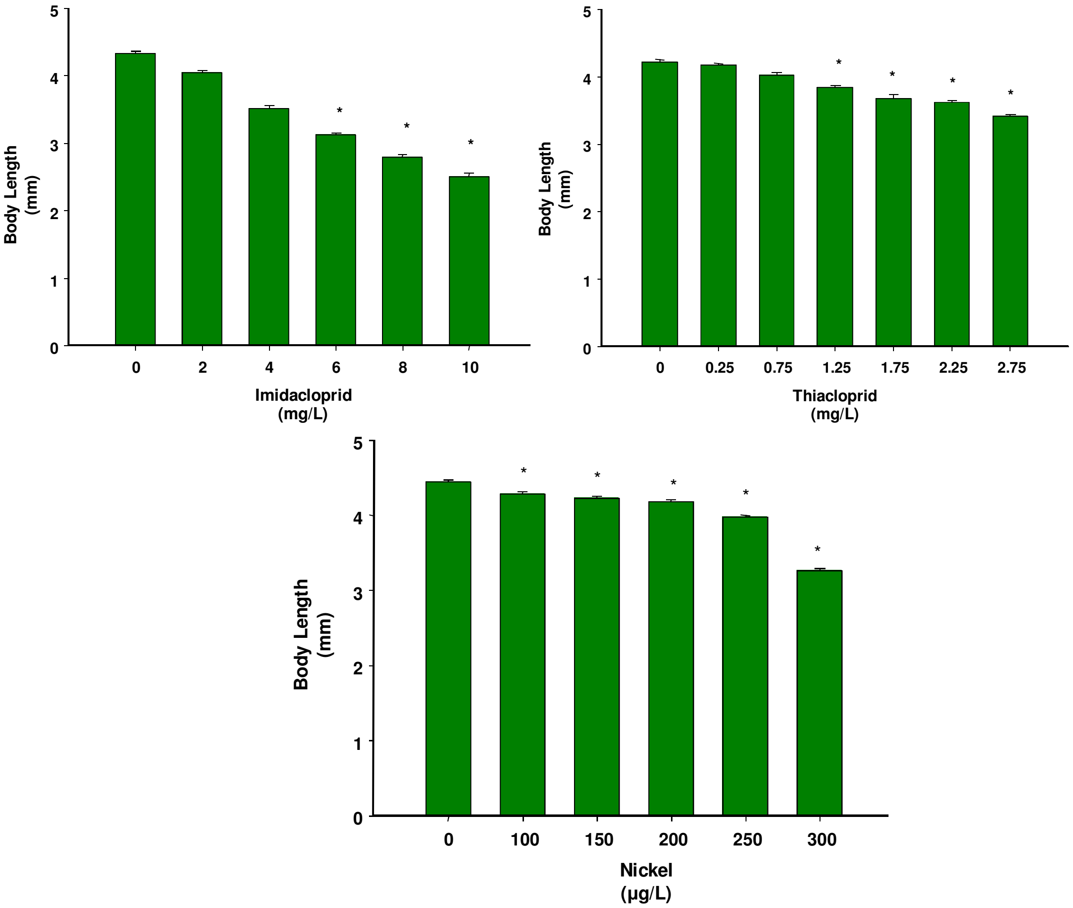

Fig.10 Mean values of the body length (and standard error) of live adult *D.magna* at the end of the test after exposure to imidacloprid, thiacloprid and nickel. (*) Denotes significant differences from control (Dunnett Method, P≤0.05).

It has been observed also that in the two highest concentrations of imidacloprid, 8 mg/L and 10 mg/L, the time for egg deposition as well as the time for the juvenile appearance has increased up to two days compared to the control as well as the other concentrations. The same has been observed for the thiacloprid exposure, but here time was increased only in one day in the two highest concentrations, 2.25 mg/L and 2.75 mg/L. However, compared to nickel there was no delay in time for the egg deposition, except for the last concentration, 350 µg/L, where the juvenile appearance was delayed one day and 100% mortality was obtained by the end of the test.

Mixture Toxicity Tests

Imidacloprid and thiacloprid are known to have similar mode of action and the Concentration Addition model would be theoretically the best model to predict their joint toxicity. As deviations from this model will be evaluated, IA model fit will also be used.

Comparing the effects of imidacloprid and thiacloprid on the reproduction of *D. magna* to the Concentration Addition model (CA) yielded a Sum of Squared Residuals (SS) of 3391.95 ($p < 0.05$). After adding parameter a to the Concentration Addition model in order to describe synergism or antagonism, SS decreased to 2993.68, although not significantly ($p(X^2) = 0.06$). Continuing in testing for deviation to dose ratio-dependency, the parameters *a* and $b_{Imidacloprid}$, (a parameter that indicates the doses where synergism changes to antagonism), it has also decreased the SS to the value of 2937.46 but again not significantly ($p(X^2) = 0.13$). However, when adding parameter *a* and b_{DL} to the dose level deviation, it decreased significantly the SS value to 2265.38, ($p(X^2) = 0.003$). Parameter *a* had a value of -2.67, which indicates synergism at low dose level and antagonism at high dose level, and the parameter *b* had a value of 0.68, which indicates that the change from synergism to antagonism occurs at a higher dose level than the EC_{50} isobole (Table 1 and Table 3).

Result on the effects of imidacloprid and thiacloprid on the reproduction of *D.magna* fitted significantly the IA model (SS = 3572.004, $p<0.05$). After adding parameter *a* to the IA equation the SS slightly decreased to 3542.45, but yet not significantly ($p(X^2) = 0.63$). Continuing in testing for deviation to dose ratio-dependency, no significant improvement was obtained on the data fit ($p(X^2) = 0.13$). However, when adding parameter *a* and b_{DL} for the dose level deviation fit, it decreased the SS to 2597.28, ($p(X^2) = 0.01$). The parameter *a* equal to -4.11, was obtained indicating synergism at low dose level and antagonism at high dose level, and the parameter b_{DL} equal to 1.45, indicates that the change from synergism to antagonism occurs at a higher dose level than the EC_{50} isobole (Table 1 and Table 3).

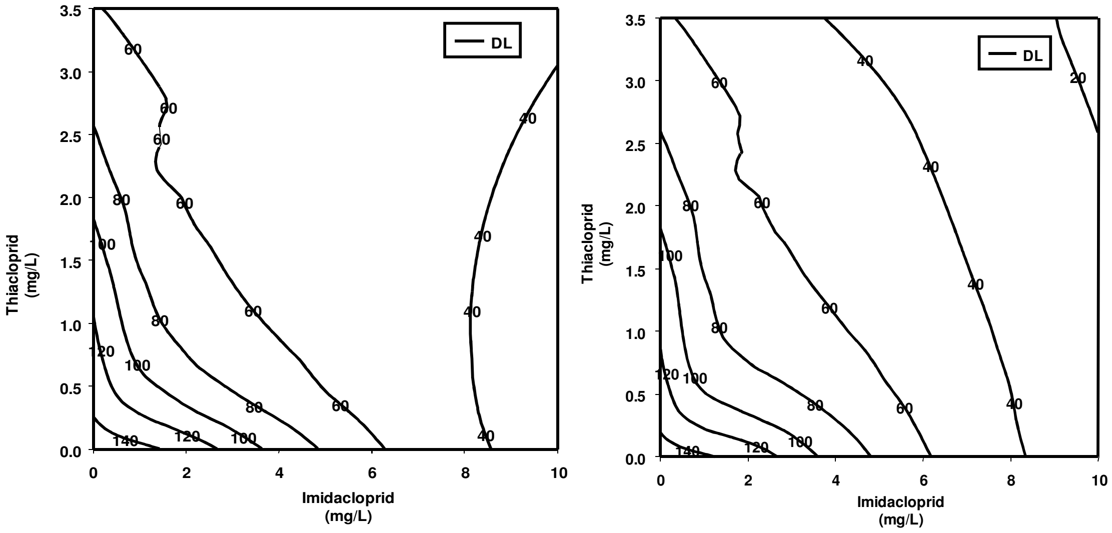

Fig.11 Concentration - response data from 21 day exposure of imidacloprid and thiacloprid showing a dose level deviation to the CA model (right) and to the IA model (left) (2D Isobolic Surface). Concentrations reported as nominal values.

Table 3

Summary of the analysis of the effect of imidacloprid and thiacloprid on the reproduction of *D. magna*. Max is the control response; β is the slope of the individual dose response curve; EC_{50} is the mean effect concentration; *a*, b_{IMI} and b_{DL} are parameters of the function; SS is the sum of squared residuals; X^2 is the test statistic; df the degrees of freedom; $p(X^2)$ indicates the outcome of the likelihood ratio test. S/A is synergism or antagonism, DR is dose ratio dependent deviation from the reference and DL is dose level deviation from the reference.

	Concentration Addition				**Independent Action**			
	Reference	S/A	DR	DL	Reference	S/A	DR	DL
max	157.66	158.39	158.46	155.14	158.64	154.88	156.36	152.69
β_{IMI}	1.63	1.59	1.53	1.94	1.43	1.56	1.65	2.01
β_{THIA}	0.87	0.88	0.89	1.57	0.71	0.72	0.48	01.55
EC_{50IMI}	4.45	4.76	4.61	4.96	4.84	5.12	4.84	4.98
EC_{50THIA}	2.26	2.60	2.71	2.67	2.50	2.80	3.49	2.76
a	-	-0.88	-1.75	-2.67	-	-0.31	-2.10	-4.11
b_{IMI}	-	-	1.51	-	-	-	3.53	-
b_{DL}	-	-	-	0.68	-	-	-	1.45
SS	3391.95	2993.68	2937.46	2265.38	3649.01	3620.98	3161.37	2677.83
X^2	-	3.50	4.03	11.30	-	0.22	4.16	8.97
df	-	1	2	2	-	1	2	2
$p(X^2)$	-	0.06	0.13	0.003	-	0.64	0.12	0.01

Though knowing that both pesticides, imidacloprid and thiacloprid, share the same mode of action, as neurotoxins that disrupt the acetylcholine receptors of the nervous system by binding to the acetylcholine receptors of postsynaptic membranes (Matsuda et al. 2001; Tomizawa and Casida 2005; Gomez-Eyles et al. 2009), we raised the hypothesis that the Concentration Addition model is the one that best describes the way the two pesticides will act when they will be found in an aquatic environment. But, it was observed a dose level dependency deviation from the Concentration Addition model, which fitted better our data and also described how the pesticides acted in this case.

For the imidacloprid and nickel mixture exposures it is expected that both chemicals have a dissimilar mode of action. As explained for the previous mixture case, both conceptual models and their deviations were tested.

Comparing the effects of imidacloprid and nickel on the reproduction of *D. magna* to the Concentration Addition model (CA) yielded a Sum of Squared Residuals (SS) of 7033.09 ($p < 0.05$). After adding parameter a to the Concentration Addition model in order to describe synergism or antagonism, SS decreased to 7031.15, although not significantly ($p(X^2) = 0.92$). Continuing in testing for deviation to dose ratio-dependency, the parameters *a* and $b_{Imidacloprid}$, (a parameter that indicates the doses where synergism changes to antagonism), it has also decreased the SS to the value of 6959.94 but again not significantly ($p(X^2) = 0.80$). No significant decrease to the SS was observed when adding parameter *a* and b_{DL} to the dose level deviation (SS = 7030.75, $p(X^2) = 0.\ 99$). (Table 1 and Table 4).

Result on the effects of imidacloprid and nickel on the reproduction of *D.magna* fitted significantly the IA model (SS = 7381.48, $p<0.05$). After adding parameter *a* to the IA equation the SS slightly decreased to 6840.08, but yet not significantly ($p(X^2) = 0.08$). Continuing in testing for deviation to dose ratio-dependency, no significant improvement was obtained on the data fit ($p(X^2) = 0.20$). No significant decrease to the SS was observed when adding parameter *a* and b_{DL} to the dose level deviation (SS = 7029.24, $p(X^2) = 0.\ 37$). (Table 1 and Table 4).

Table 4

Summary of the analysis of the effect of imidacloprid and nickel on the reproduction of *D. magna*. max is the control response; β is the slope of the individual dose response curve; EC_{50} is the mean effect concentration; *a, b_{IMI}* and *b_{DL}* are parameters of the function; SS is the sum of squared residuals; X^2 is the test statistic; df the degrees of freedom; $p(X^2)$ indicates the outcome of the likelihood ratio test. S/A is synergism or antagonism, DR is dose ratio dependent deviation from the reference and DL is dose level deviation from the reference.

	Concentration Addition				**Independent Action**			
	Reference	S/A	DR	DL	Reference	S/A	DR	DL
max	142.73	142.86	143.44	142.67	155.20	145.66	145.91	147.04
β_{IMI}	1.31	1.31	1.29	1.32	1.04	1.28	1.28	1.10
β_{THIA}	2.98	2.94	2.93	2.98	1.86	2.54	2.51	2.20
EC_{50IMI}	6.97	7.03	7.20	7.04	5.43	6.98	6.96	6.69
EC_{50Ni}	0.31	0.31	0.30	0.31	0.30	0.31	0.31	0.32
a	-	-0.05	0.33	-0.09	-	-1.19	-1.12	0.001
b_{IMI}	-	-	-0.89	-	-	-	-0.09	-
b_{DL}	-	-	-	0.58	-	-	-	1290.41
SS	7033.09	7031.15	6959.94	7030.95	7381.48	6840.08	6839.64	7029.24
X^2	-	0.01	0.43	0.01	-	3.12	3.13	2
df	-	1	2	2	-	1	2	2
$p(X^2)$	-	0.91	0.81	0.99	-	0.08	0.21	0.37

Both models showed to fit our data, so we can say that imidacloprid and nickel act jointly.

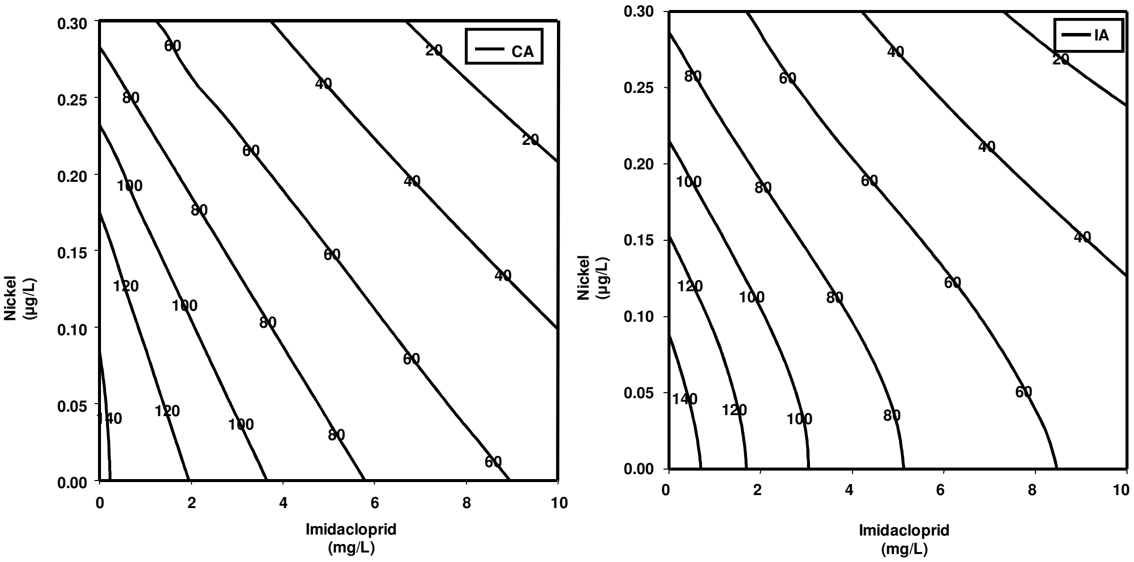

Fig.12 Concentration - response data from 21 days exposure of imidacloprid and nickel showing no deviation to the CA model (right) or to the IA model (left) (2D Isobolic Surface). Concentrations reported as nominal values.

Discussion – Conclusion

Single Compound Chronic Toxicity Tests

There are few studies on the neonicotinoid pesticides effects, such as imidacloprid and thiacloprid, to *D. manga*. And even those, are not referring to the effects of those chemicals to the whole life cycle of the organism but to effects caused either by 24, 48 or 96 hours of exposure.

According to a comparative study between imidacloprid and its commercial liquid formulation, LOEC values ranging from 2.5 mg/L to 10 mg/L was observed after a 21 days exposure period, (Jemec et al. 2007) which is in accordance with our findings and according to Young and Blakemore (1990), with a LOEC value of 7.3 mg/L for immobilization was assessed. The acute toxicity of imidacloprid to the cladoceran *D. magna* has been confirmed as relatively low (Tomlin 2002; Sánchez-Bayo and Goka 2006; Kungolos et al. 2009) where LC_{50} = 64 mg/L – 85 mg/L values have reported (Fossen 2006).

When comparing chemicals' toxicity, nickel was the most toxic to *D. magna* (EC_{50} = 289.29 µg/L) followed by thiacloprid (EC_{50} = 2.45 mg/L) and imidacloprid (EC_{50} = 5.5 mg/L).

Beketov and Liess (2008) presented a value for thiacloprid (EC_{50} = 4.74 mg/L for reproduction) during 4, 14 and 30 days of exposure of *D. magna*. The difference in EC_{50} values between the above mentioned results and our results can be attributed to the method they followed to obtain this value. The OECD guideline 211 was adapted for the test and the organism was only exposed for 24 hours to different concentrations of thiacloprid (in our test the organism was exposed for 21 days) and then transferred to a clean medium until the end of the test. Another parameter to this difference can be the different clone that they have used (clone B) when compared to the clone used in this study (clone K6). Different clones of the cladoceran *D.magna* show different tolerance to the same chemicals (Münzinger and Monicelli 1991; Soares et al. 1992; Chenon et al. 2000).

Daphnia magna is considered to be very sensitive to several heavy metals, such as Hg, Cu, and Cr which are the most toxic to the organism, as well as Ni (Fargasova 1994; Burba 1999). In this study and as mentioned above nickel appears to be the most toxic when compared to the two insecticides. But yet when compared to other studies the EC_{50} value obtained here appears to be higher than the ones attributed in other studies (Deleebeeck et al. 2007). The medium hardness affects the toxicity of the cationic metals such as nickel by lowering it and the fact tests in this study were carried out in ASTM hard water (> 140 mg/L $CaCO_3$) and organisms tested according to Deleebeeck (2007) were exposed to nickel in a lower hardness medium (43.4 mg/L) can explain the difference in results. It was also observed that during the 21-days of exposure of *D. magna* to nickel the two lowest concentrations showed a stimulation on the reproduction effort of the organism which was also reported by Winner and Farrell (1976) and Dave (1984) cited by Burba (1999) when daphnids were exposed to Cu. Another EC_{50} value was reported by Pane et al. (2004) considerably lower than the one obtained in this study after 21-days of exposure (EC_{50} = 85 µg/L). The different hardness of water exposure between the two studies may also preside for the different reproduction numbers, as Pane et al. (2004), exposed the organisms in hardness equal to 45 mg/L (as $CaCO_3$). These authors also reported a not significant increase of the number of neonates produced per breeding animal after 21-days in concentration lower than the EC_{50} (equal to 42 µg/L), a pattern that occurred in this study as well. This increase can be due to the fact that nickel is essential for certain biological and physiological processes in aquatic organisms (Muyssen et al. 2004; Chowdhury et al. 2008) as in various enzymes in (cyano)bacteria and plants (Muyssen et al. 2004).

Mixture Toxicity Tests

As far as we know, till now there is no published data on response patterns of *D. magna* exposed to the same pesticide mixture, but there are data from other species, such as *Caenorhabditis elegans* and *Eisenia fetida,* where the joint effect of imidacloprid and thiacloprid was evaluated. For *C. elegans,* the dose level-dependent deviation from the concentration addition model described the effects of the mixture significantly better while effects of the mixtures to the *E. fetida*, the reference model fitted the data since there was no significant improvement (Gomez-Eyles et al. 2009). It has been reported that studies with similar acting chemicals show that the Concentration Addition model demonstrates a good ability in predicting the joint effect of those chemicals with similar mode of action but yet there have been cases where deviations from this model have occurred when those chemicals were tested (Deneer 2000; Jonker et al. 2005; Gomez-Eyles et al. 2009). The Concentration Addition model underestimated the toxicity of the two pesticides when they were in concentrations lower than the EC_{50}, while in concentrations higher than the EC_{50} of the mixture the model showed to have overestimated the toxicity of the binary mixture of pesticides.

On the other hand, when we talk about the mixture of the pesticide, imidacloprid with the heavy metal, nickel, we can only assume that they have a dissimilar mode of action. Imidacloprid is a neurotoxin that acts as an agonist at the acetylcholine receptors (nAChRs) of insects. Nickel exposure to *D.magna* has shown that it significantly affects the Mg^{+2} homeostasis, reduces the oxygen consumption rate as well as the haemoglobin concentration (Pane et al. 2003), something that Vandenbrouck (2009) also agrees. According to Vandenbrouck (2009), daphnids that were exposed previously to nickel showed effects on several gene classes, involved in different metabolic processes (protein and chitin related processes). Bearing in mind the different effects that they cause, we therefore, assume that they follow and act in different biological paths when entering in the organism. When testing stressors with different modes of action, the Independent Action model is the one that best describes their joint effects which was the one that fitted the data from this mixture with no deviation.

It was observed, that daphnids stayed often in the medium surface (due to antennae movement), which made them feed improperly, both in the single as well as in the mixture tests.

Concluding, risk assessment is usually conducted by focusing on the toxicity of individual chemicals, while we are facing contamination of an area by far more than one chemical in the majority of the cases (Backhaus et al. 2003). The use of binary mixtures to assess the risk from hazardous chemicals that will end up in the environment, and in this case in the aquatic environment, it will provide information and evaluate the way the chemicals act, the possible effects they may cause to the organisms and to the system’s ecology.

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Chapter III

Effects of binary combinations of chemicals and natural stressors to *Daphnia magna*

Effects of binary combinations of chemicals and natural stressors to *Daphnia magna*.

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Abstract

In aquatic ecosystems several stressors may act together and affect the life traits of organisms. Pesticide runoffs are usually associated with high inputs of organic matter and depletion of oxygen in aquatic systems. This study aimed to combine chemicals and different algae concentrations and evaluate their joint effect to the life traits of *Daphnia magna*. The neonicotinoid insecticide imidacloprid and a heavy metal nickel chloride were used and a 21d chronic test was carried out to obtain reproduction and body length data. Results show an increase in the reproduction and body length of *D. magna* as food level increases. Whenever possible, the conceptual model Independent action, usually used for assessing response patterns in chemical mixtures, was also used to fit our data. It was observed that food availability changed the toxicity of imidacloprid and nickel chloride.

Introduction

In aquatic ecosystems several stressors may act together and affect the life traits of organisms. Pesticides and metallic elements can be frequently found in the aquatic environment, in combination with several natural stressors such as different food levels, temperatures, dissolved oxygen depletion, UV radiation, among others. Pesticide runoffs are usually associated with high inputs of organic matter, nutrients and depletion of oxygen in aquatic systems. Imidacloprid is a neonicotinoid insecticide widely used in crops and slowly taking over the market of other competing organophosphorous pesticides (Jemec et al, 2007). Domestic wastewater effluents (mostly As, Cr, Cu, Mn and Ni) and non – ferrous metal smelters (Cd, Ni, Pb and Se) are considered to be the major sources of trace metal pollution in aquatic ecosystems (Cempel and Nikel 2006). Nickel is an abundant heavy metal throughout the planet and it is essential in many forms of life. Nickel metal and its alloys are used widely in the metallurgical, chemical and food processing industries. Nickel chloride, sulphate, nitrate, carbonate, hydroxide, acetate and oxide are nickel salts of great commercial importance (Grandjean 1984; Clarkson 1988; Cempel and Nikel 2006). According to Barceloux et al. (1999), the main sources of exposure of nickel for the general population are through the drinking water and food giving as an example that an average American diet contains about 300 µg Ni/d.

Daphnia populations can periodically be found in the environment under conditions of natural stress such as no food, low (Antunes et al. 2003; Pieters and Liess 2006) or high food levels. Algae concentrations up to x10^6 cells/ml can be found in eutrophic environments, and therefore studies using algal densities are suggested in order to evaluate the dynamics of toxicants in polluted ecosystems, (Rodgher and Gaeta Espíndola 2008) or low and high levels of temperature (Rinke and Petzoldt 2003) that can affect among others species growth, reproduction and survival. According to some studies (Buikema et al. 1980; McCauley et al. 1990; Pieters and Liess 2006) low food concentrations occur in the environment and they are able to alter the sensitivity of organisms, like *Daphnia*, to contaminants (Chandini 1988; Chandini 1989; Antunes et al. 2003; Pieters and Liess 2006).

Several studies have taken place throughout the years focusing on the toxicity of several chemicals to several organisms when tested in different food conditions Barry et al. (1995) reports that the toxicity of esfenvalerate, a synthetic pyrethroid, to *Daphnia carinata* has increased as the concentration of the food decreased while the opposite happened when the toxicity of endsulfan, an organ chlorine pesticide, increased in higher food concentrations. It was also reported an increasing sensitivity of *Daphnia magna* and *Daphnia* cf. *longispina* clones to the toxicant stress (the herbicide propanil), when food supply decreases (Pereira and Gonçalves 2007; Pereira et al. 2007). Antunes et al. (2004) reported lindane as toxic to daphnids depending on the food level, while it suggested that further studies needed to be carried out using food as an important factor for evaluating the toxicity of chemicals. Fliedner (1997) supports that acute toxicity to lindane appears to be higher when the organism is being exposed to contaminated algae, since the exposure happens via food, which is of high relevance for daphnids. When it comes to metals, dietary exposure may occur due to their being adsorbed to and taken up by algae before the organisms ingest them through filtering processes (Rodgher and Gaeta Espíndola 2008). Another study has shown that the toxicity of Cr(VI) to *D.magna* is significantly different when comparing exposed organisms to different food levels, making the metal more toxic in low food concentrations and less toxic as the concentration increases (Gorbi et al. 2002). In addition, and according to Koivisto et al. (1992) cited by Gorbi et al. (2002), different species of cladoceran showed less resistance to copper when exposed to low concentrations of food and suggested that the difference may be due to metal adsorption to the algae or to lower tolerance to copper when exposed to starvation conditions. A study based on the energy status of daphnids after being exposed to Cd concentrations, shows that in higher food concentrations, the toxicity of Cd is more evident (Smolders et al. 2005).

This study aimed to evaluate the joint effects of chemicals (imidacloprid and nickel chloride) at different algae concentrations to the life traits of *Daphnia magna,* a non-target organism. In addition to the calculation of ECx endpoints to compare toxicities, the independent action model will be used to fit our data and predict the joint toxicity of combined stressors. This approach has already been used by other authors with accurate results (e.g. Ferreira et al. 2008; Long et al. 2009). Additionally, deviations from the conceptual model for synergism or antagonism patterns will be also tested.

Materials and Methods

Test Organism

All experiments were performed using the cladoceran *Daphnia magna* (Fig.8), clone K6 (Antwerp, Belgium) as test organism, which has successfully being cultured at the laboratory from more than a year. Long-term bulk cultures of *D. magna* were maintained in artificial medium, ASTM hard water (ASTM 1998). Glass aquariums (6L capacity) were kept with ASTM hard water, and daphnids were fed with the algae *Pseudokirchinella subcapitata* in a ratio of $3x10^5$cells/ml and an organic additive (Marinure seaweed extract, supplied by Glenside Organics Ltd.) (Baird et al. 1989) at a ratio of 6ml per litre of ASTM medium. The cultures were maintained in a controlled temperature chamber at 20ºC at a 16:8 light: dark cycle and culture medium was renewed three times a week. New cultures of daphnids were initiated using the neonates from the third to the fifth brood of the old cultures. According to the OECD procedure, a reference test with potassium dichromate ($K_2Cr_2O_7$) was performed for testing the sensitivity of the daphninds.

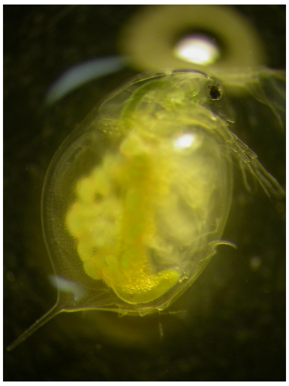

Fig.8. *Daphnia magna* Straus (Clone K6)

Test Chemicals

Chemical compounds used in this study were the neonicotinoid insectide imidacloprid (CAS No. 138261-41-3, Bayer, Germany) and the heavy metal nickel (II) chloride hexahydrate (CAS No. 7791-20-0, Merck, Germany).

Exposure medium contamination was controlled by chemical analysis by high-pressure liquid chromatography with UV detector (HPLC – UV) for imidacloprid and by inductively coupled plasma-mass spectrometry (ICP-MS) for nickel. To control medium contamination for imidacloprid, chemical analysis was performed to two samples of the lowest and the highest concentrations and two samples of the stock solution, due to the complex experimental design used for mixture experiments. For nickel, the same chemical analysis was performed as described for the insecticides.

Chronic Toxicity Tests with Chemicals: Chronic toxicity was assessed for each chemical compound using the OECD 211 guideline, for the *Daphnia magna* reproduction test (OECD 211 Guideline 2008).

Five concentrations for imidacloprid and six for the nickel, plus a negative control, with 10 replicates of one animal (<24 hours old) each were used for the experimental setup. Tests were maintained in a chamber at 20ºC at a 16:8 light: dark cycle, fed daily with a ratio of $3x10^5$ cells of algae per ml per day and the medium was renewed every other day for 21 days. Reproduction was reported and neonates were removed from the vessels. Each vessel was recorded daily for mortality of the organisms and the mortality of the parent animals did not exceed 20% at the end of the test.

Values for pH, dissolved oxygen and temperature were obtained weekly to check for the validity criteria. At the end of the test the total number of offspring produced per parental animal was calculated and the adult daphnids were measured. Adult daphnids at the end of the test were placed under a stereo – microscope and the body length of each daphnid from the point immediately above the eyespot to the base of the dorsal spine was registered.

The nominal concentrations for imidacloprid ranged from 2mg/l to 10mg/L and for nickel from 100µg/L to 350µg/L.

Chronic Toxicity Tests with Natural Stressor: The OECD 211 guideline for chemical testing, for *Daphnia magna* (OECD 211 Guideline 2008) was adopted and adapted in this test to evaluate the effects of different food levels in the reproduction and body length.

Low and high food concentrations were tested ranging from starvation to $2x10^5$ cells of algae per ml of medium and from $4x10^5$ to $6x10^5$ cells of algae per ml of medium, respectively with a control of $3x10^5$ cells/ml (the concentration of food usually added to *D. magna* cultures).

Tests were maintained in a controlled chamber at 20ºC at a 16:8 light: dark cycle, fed everyday with the corresponding amount of food to each food level and the medium was renewed every other day for 21 days. Reproduction was reported and neonates were removed from the vessels. Each vessel was recorded daily for mortality of the organisms.

Values for pH, dissolved oxygen and temperature were obtained weekly to check for the validity criteria. At the end of the test the total number of offspring produced per parental animal and their body length were recorded.

Binary Combination Toxicity Tests

In the combined toxicity tests with the two chemicals and the natural stressor, the number of replicates per each treatment was decreased from ten to one to allow the use of more treatments per test, in order to obtain a broader range of the chemical's response to the combination. In both combination sets, imidacloprid – food levels, and nickel – food levels, an experimental factorial design was used. For imidacloprid in combination with food levels, concentrations of food ranged from 0 to $6x10^5$ cells of algae per ml of medium and imidacloprid concentration from 2 mg/L to 10 mg/L; for Nickel – food levels combination, concentrations of food ranged also from 0 to $6x10^5$ cells of algae per ml of medium and nickel concentrations from 100 µg/L to 300 µg/L. The food level approach was divided in two sets, when in combination with chemicals exposure: low food level ranging from 0 to $3x10^5$ cells of algae per ml of medium and high ranging from 3 to $6x10^5$ cells of algae per ml of medium from using $3x10^5$ as control. To obtain a concentration–response curve for the statistical analysis within stressors combination experimental set up, where the toxicity increases as concentrations increase, a transformation of [3 – x] was made, where 3 corresponds to the control concentration (3×10^5 cells/ml of food) and x is the real concentrations tested (0, 0.5, 1, 1.5 and 2×10^5 cells/ml of food), while for the high food level no transformation was made to the concentrations.

Data Analysis

The EC_{50} values obtained from each single chemical or natural stressor exposure (imidacloprid, nickel and low food concentration) to *D.magna* were calculated by fitting the data to the logistic model, $Y_{(ci)} = Y_{max} / [1 + (c_i / EC_{50})^{\beta}]$, with a $Y_{(ci)}$ response as a function of the maximum response Y_{max}, the exposure concentration c_i, the EC_{50} value and the slope β of the response curve, a non linear regression, sigmoidal logistic three parametric curve using the software SigmaPlot, vers.10.0. For the NOEC and LOEC values of each of the chemicals and the natural stressor, a One Way Analysis of Variance (ANOVA) and the multiple comparisons Dunnett's Method were used to detect differences between data that followed a normal distribution (Sigma Stat software).

For the prediction of the joint toxicity of the combined stressors (only in low food levels), the conceptual models, Concentration Addition (CA) and Independent Action (IA), were used (Jonker et al. 2005). The two models and their deviations (Synergism/Antagonism, Dose Level Dependency, and Dose Ratio Dependency) were compared using the method of maximum likelihood and the best fit was chosen (Table 1).

For the high food levels the EC_{50}, the NOEC and LOEC values were not possible to calculate, although there was a dose-response increase when food levels increased. But no EC_{50} could be calculated. EC_{50} values of nickel to each food level were calculated by a probit analysis using the software Minitab.

Table 1

Interpretation of additional parameters (*a* and *b*) that define the functional form of deviation pattern from concentration addition (CA) and independent action (IA); adapted from Jonker et al. 2005.

Deviation Pattern	**Parameter *a* (CA and IA)**	**Parameter *b* (CA)**	**Parameter *b* (IA)**
synergism/antagonism (S/A)	**a>0:** antagonism **a<0:** synergism		
Dose-ratio dependent (DR)	**a>0:** antagonism except for those mixture ratios where negative b value indicate synergism	**b_i >0:** antagonism where the toxicity of the mixture is caused mainly by toxicant *i*	
	a<0: synergism except for those mixture ratios where positive b value indicate antagonism	**b_i <0:** synergism where the toxicity of the mixture is caused mainly by toxicant *i*	
Dose-level dependent (DL)	**a>0:** antagonism low dose level and synergism high dose level	**b_{DL}>1:** change at lower EC50 level	**b_{DL}>2:** change at lower EC50 level
		b_{DL}=1: change at EC50 level	**b_{DL}=2:** change at EC50 level
	a<0: synergism low dose level and antagonism high dose level	**0<b_{DL}<1:** change at higher EC50 level	**1<b_{DL}<2:** change at higher EC50 level
		b_{DL}<1: No change but the magnitude of S/A is DL dependent	**b_{DL}<1:** No change but the magnitude of S/A is effect level dependent

Results

Single Stressors Chronic Toxicity Tests

According to the OECD guideline 211 (OECD 211 Guideline 2008), the survival, reproduction and body length of the adult *D. magna* were assessed after 21 days of exposure to each chemical and natural stressor. The reproduction is expressed as the cumulative number of offspring per live *D. magna* after exposure and the results are shown in Table 5.

Table 5

Toxicity values from 21-days reproduction tests with *D. magna* exposed to imidacloprid, nickel and different food levels. **n.d.- not determined*

Chemicals	**EC_{50}**	**Std. Error**	**R^2**	**NOEC**	**LOEC**
Imidacloprid (mg/L)	5.5	0.23	0.92	2	4
Nickel (µg/L)	289.29	3.08	0.86	200	250

Natural Stressor	**EC_{50}**	**Std. Error**	**R^2**	**NOEC**	**LOEC**
Low Food ($x10^5$ cells/ml)	1.79	0.04	0.95	1	1.5
High Food ($x10^5$ cells/ml)	n.d.	n.d.	n.d.	n.d.	n.d.

Reproduction was suppressed significantly as the concentrations of all three chemicals were increased. EC_{50} values for chemicals and natural stressors exposures are depicted on Table 5.

For the imidacloprid exposure, a 10% of mortality occurred in the highest concentration (10mg/L). For nickel exposure and after 15 days of exposure to 350 µg/L (highest concentration) 100% of mortality was observed and a 10% and 20% of mortality occurred in the concentrations of 250 µg/L and 300 µg/L, respectively, by the end of the test. No mortality occurred in the control of any of the tests.

When it comes to the natural stressor, it was observed that as the food level was increased an increase on the reproduction rate was observed; on the other hand when the food levels decreased, the reproduction rate decreased, and when there was no food, daphnids did not reach maturity. In the daphnids exposed to starvation conditions, no eggs were observed during the 21-days test in their pouch and therefore, no offspring production. In terms of age of first reproduction, daphnids exposed to low food levels than the control were significantly delayed while in higher concentrations of food the 1st brood was release was in accordance to what happened in the control. Significant differences were observed in the number of broods in the lower food levels when compared to the control. No mortality occurred in any of the different food levels.

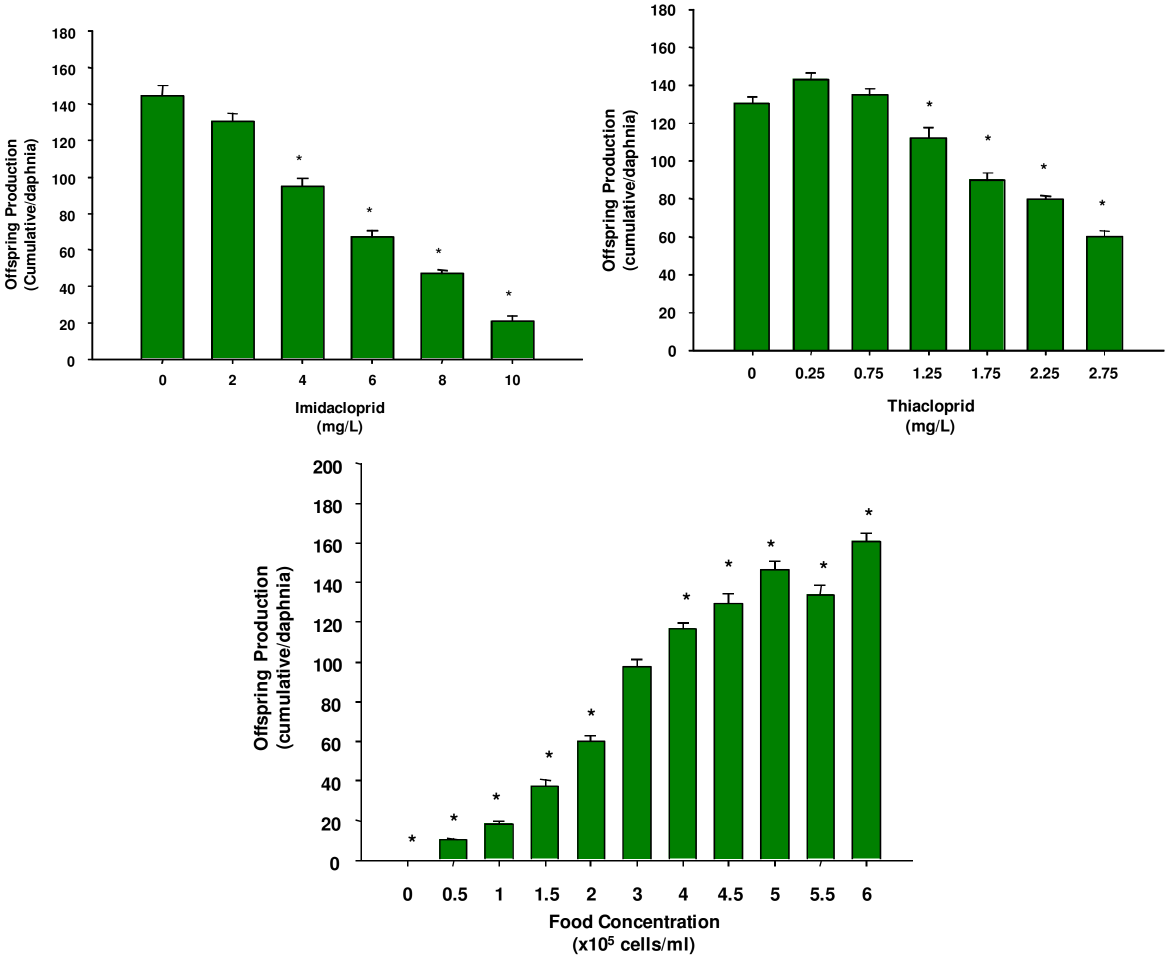

Fig.13 Mean cumulative number of offspring production (and standard error) per live *Daphnia magna* exposed to imidacloprid, nickel, and food levels. (*) Denotes significant differences to the respective control (Dunnett Method, P≤0.05). The control situation in the food levels experiment was 3x10^5 cell/ml.

Body length of the adult daphnids was also affected significantly but only for imidacloprid exposure (One Way Anova, $F_{5,52}$= 303.46, P<0.001), while for nickel no significant difference between treatments was observed (One Way Anova, $F_{5,50}$=178.41, P<0.001). The size of daphnids exposed to starvation situations (0x10^5 cells/ml) was significantly different when compared to the size of the daphnids from the control (One Way Anova, $F_{5,59}$= 14.91, P<0.001).

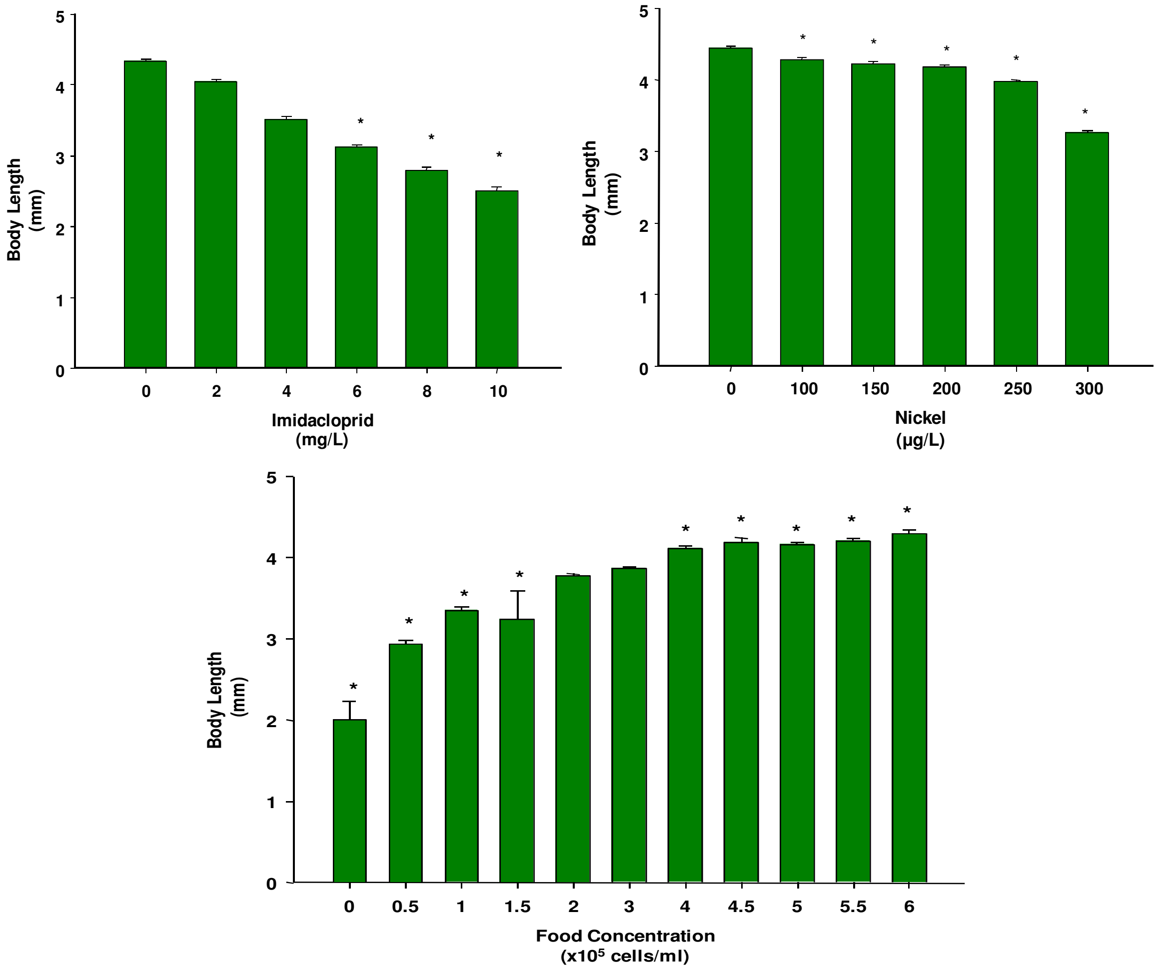

Fig.14 Mean values of the body length (and standard error) of live adult *D. magna* in the end of the test after exposure to imidacloprid, nickel and food levels. (*) Denotes significant differences to the respective control (Dunnett's Method, P≤0.05). The control situation in the food levels experiment was 3x10^5 cell/ml.

Binary Combination Toxicity Tests

According to the OECD guideline 211 (OECD 211 Guideline 2008), the survival and reproduction of the adult *D. magna* were assessed after 21 days of exposure to each chemical and to their mixture.

Conceptual models have been described to predict the joint toxicity of chemical mixtures: Concentration Addition and Independent Action, which are usually based and chosen considering the mode of action of each stressor. If the stressors have similar mode of action then the Concentration Addition model is chosen (Loewe and Muischnek 1926), while when they have dissimilar mode of action the Independent Action model is preferred (Bliss 1939). In those cases where the mode of action of stressor(s) is unknown, both models are used and the best fit evaluated.

In this approach modes of action of the three stressors were considered different, although few is know about low/high food mode of acting on an organism. Also, we only used this approach for the combined effects of chemicals and low food concentrations because no EC_{50} values or dose-response curve (for deleterious effects) could be found for high food levels.

Result on the effects of imidacloprid with low food levels on the reproduction of *D. magna* fitted significantly the IA model (SS = 3348.87, $p<0.05$). After adding parameter *a* to the IA equation the SS slightly decreased to 3342.94, but yet not significantly ($p(X^2) = 0.84$). Continuing in testing for deviation to dose ratio-dependency, no significant improvement was obtained on the data fit ($p(X^2) = 0.96$). No significant decrease to the SS was observed when adding parameter *a* and b_{DL} to the dose level deviation (SS = 3188.77, $p(X^2) = 0.56$). (Table 1 and Table 6).

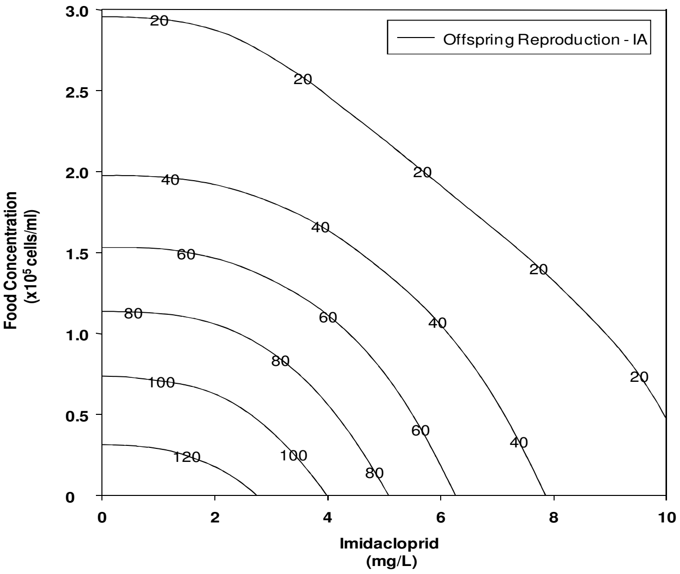

Fig.15 Concentration - response data from 21 days exposure of imidacloprid and low food level showing no deviation to the IA model (2D Isobolic Surface). Control value for food level is $3x10^5$ cells/ml. Concentrations reported as nominal values.

No reproduction was observed when daphnids were exposed to starvation situations ($0x10^5$ cells/ml) when combined with imidacloprid. When combining starvation and the highest concentrations of imidacloprid, daphnids died after the first week of the test.

Table 6

Summary of the analysis of the effect of imidacloprid and low food concentrations on the reproduction of *D. magna*. max is control response; β is the slope of the individual dose response curve; EC_{50} is the median effect concentration; a, b_{IMI} and b_{DL} are parameters of the function; SS is the sum of squared residuals; X^2 is the test statistic; df the degrees of freedom; $p(X^2)$ indicates the outcome of the likelihood ratio test. S/A is Synergism/Antagonism, DR is dose ratio dependent deviation from the reference and DL is dose level deviation from the reference.

	Independent Action			
	Reference	S/A	DR	DL
Max	134.86	135.59	134.94	135.54
β_{IMI}	2.82	2.80	2.60	2.84
β_{Food}	2.16	2.14	2.33	2.21
EC_{50IMI}	5.79	5.72	5.85	5.71
EC_{50Food}	1.32×10^5	1.29×10^5	1.26×10^5	1.30×10^5
A	-	0.16	2.10	-0.34
b_{IMI}	-	-	-4.25	-
b_{DL}	-	-	-	2.01
SS	3348.87	3342.94	3337.28	3188.77
X^2	-	0.04	1.18	0.08
Df	-	1	2	2
$p(X^2)$	-	0.84	0.56	0.96

Result on the effects of nickel with low food levels on the reproduction of *D. magna* fitted significantly the IA model (SS = 3543.56, $p<0.05$). After adding parameter *a* to the IA equation the SS slightly decreased to 3398.78, but yet not significantly ($p(X^2) = 0.28$). Continuing in testing for deviation to dose ratio-dependency, no significant improvement was obtained on the data fit ($p(X^2) = 0.45$). No significant decrease to the SS was observed when adding parameter *a* and b_{DL} to the dose level deviation (SS = 3333.86, $p(X^2)$ = 0. 43) (Table 1 and Table 7).

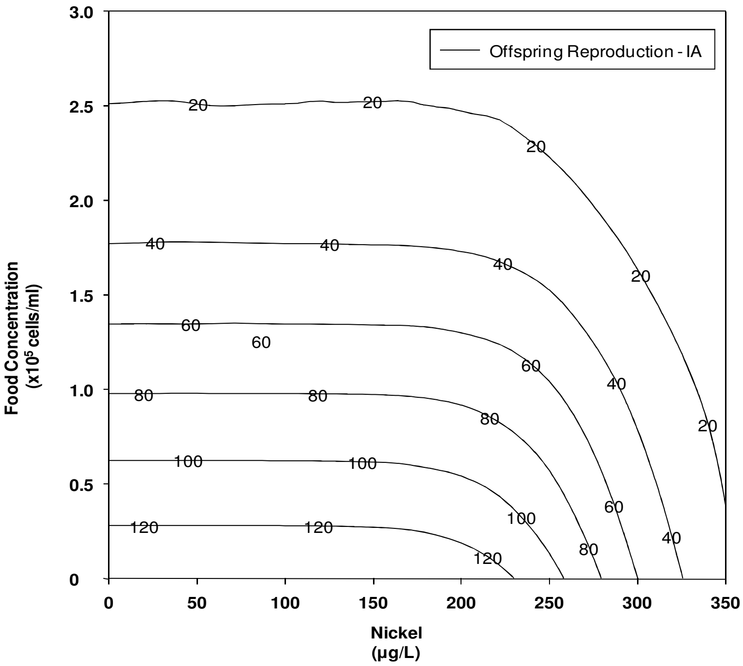

Fig.16 Concentration - response data from 21 days exposure of nickel and low food level showing no deviation to the IA model (2D Isobolic Surface). Control value for food level is $3x10^5$ cells/ml. Concentrations reported as nominal values.

No reproduction was observed when daphnids were exposed to starvation situations ($0x10^5$ cells/ml) and nickel. When combining starvation and the three highest concentrations of nickel, 200 µg/L, 250 µg/L, 300 µg/L and 350 µg/L, daphnids died after the second week of the test.

Table 7

Summary of the analysis of the effect of nickel and low food concentrations on the reproduction of *D. magna*. max is control response; β is the slope of the individual dose response curve; EC_{50} is the median effect concentration; *a*, b_{IMI} and b_{DL} are parameters of the function; SS is the sum of squared residuals; X^2 is the test statistic; df the degrees of freedom; $p(X^2)$ indicates the outcome of the likelihood ratio test. S/A is Synergism/Antagonism, DR is dose ratio dependent deviation from the reference and DL is dose level deviation from the reference.

	Independent Action			
	Reference	S/A	DR	DL
max	136.29	137.51	137.91	137.97
β_{Ni}	8.43	8.33	8.44	8.66
β_{Food}	2.18	2.10	1.98	2.38
EC_{50Ni}	291.63	287.83	285.87	285.28
EC_{50Food}	1.15×10^5	1.02×10^5	1.04×10^5	1.07×10^5
a	-	0.79	-0.42	-0.83
b_{Ni}	-	-	2.80	-
b_{DL}	-	-	-	2.79
SS	3543.56	3398.78	3344.39	3333.86
X^2	-	1.17	1.62	1.70
df	-	1	2	2
$p(X^2)$	-	0.28	0.45	0.43

The concentrations of imidacloprid and nickel that cause 50% of the offspring reproduction to decrease was assessed in accordance to each different food level and the values are seen in **Table 8**.

The EC_{50} values for the imidacloprid and nickel exposure at different food levels increased with the increase of food (just for food levels > $3x10^5$). Although this might mean a decrease in toxicity, the values obtained for the standard errors makes us cautious when concluding on changes in toxicity.

On the other hand, when low food levels were present with imidacloprid and nickel, values for EC_{50} decreased.

Table 8.

EC$_{50}$ values for *D. magna* exposed to imidacloprid and nickel at different food concentration levels (endpoint-reproduction as number of neonates).

	EC$_{50}$ (st. error)						
Food (x10^5 cells/ml)	0	1	2	3	4	5	6
Imidacloprid (mg/L)	<2	5.61	5.80	5.65	7.75	6.16	6.30
		(4.35)	(0.74)	(0.32)	(18.59)	(0.21)	(5.81)
Nickel (μg/L)	<100	278.91	310.90	282.09	273.55	256.67	290.35
		(19.74)	(26.09)	(7.87)	(30.74)	(30.58)	(18.57)

Discussion – Conclusion

Single Stressors Chronic Toxicity Tests

In this study the results show that both size and fecundity/reproduction of the parental animal were significantly affected by different food levels and the chemicals exposures. Offspring were produced even at low food concentrations (0.5 x 10^5 cells/ml), though the reproduction and body length of the daphnids was significantly different when compared to the control indicating that that the food level was not adequate to maintain a metabolic rate close to its normal.

According to Enserink et al. (1990) and Smolders et al. (2005), in low concentrations *D. magna* allocate the energy from growth and increase reproduction to smaller female daphnias that are releasing smaller broods but with bigger and more resistant offspring (Enserink et al. 1993; Smolders et al. 2005), as well as in survival and longevity of the species turning them less sensitive to possible stress. The same author supports that in higher food concentrations, *D. magna* allocates its energy budget in growth and reproduction meaning that the survival and longevity becomes less important since they have already assured the success of the population making them less tolerant to any chemical or natural stress (Smolders et al. 2005).

Binary Combination Toxicity Tests

The results from this study show a higher toxicity to imidacloprid in *D. magna* when the concentrations of food in the combination test were lower than the control while for the nickel the toxicity seems only affected in starvation conditions. This behaviour of the chemicals can be discussed based on possible changes in availability of the chemicals when added in the medium, whether being adsorbed to the algae or if they are available as free ions.

Daphnids exposed to imidacloprid showed an intense antennae movement, which kept them either close to the surface, away from algae deposition in the bottom, or in the bottom of the vial in a continues "spinning" motion. This behaviour may be a result of the binding of the insecticide to the acetylcholinasterase receptors of the daphnid resulting in the accumulation of acetylcholine and in not coordinated movements as the above mentioned, a behaviour that was also described by Dodson and Hanazato (1995) as a result of carbaryl exposure to cladocerans.

Daphnia avoid predation in lakes by migrating downward into dark waters during the day, where shortage of food and the presence of other stressors (e.g. oxygen depletion) occurs, and upwards during the afternoon in order to feed (Hanazato 2001). According to Hanazato (2001), due to the diel vertical migration, daphnids are exposed to low food concentrations, among other stressors, resulting in increased sensitivity to pesticides that can also be part of the stressors already mentioned.

According to De Schamphelaere (2004) and Rodgher (2008), metals can be adsorbed to the algae cells, becoming more or less available to the daphnids through food.

Nickel tends to adsorb to the algae cells making it available through food consumption. As a result, low concentrations of food would result in low intake rate of nickel through food, therefore lower toxicity of the heavy metal nickel to the organism *D. magna* was expected. But, from the EC_{50} values obtained, no significant changes were observed in this endpoint when comparing the control and the other low food concentrations (excluding the starvation case). The opposite could be suggested as the concentrations of food increases, the ingestion of the metal from the organism will increase since higher number of algae cells would be consumed. Again, and from the same endpoints this was not observed. So this suggests another route for nickel's uptake by the daphnids. When chemical analysis was performed no alterations on the nickel concentrations was observed in the aqueous solutions due to algae existence, concluding that the uptake of the metal from the daphnids through food was low. The differences observed in the daphnids between the food and nickel concentrations can be attributed to the differences the daphnids have in the physiology and reproductive strategy they follow. However, the effects of nickel on survival and reproduction of *D.magna* were determined by the food concentrations. A similar study on different food concentrations and different levels of cadmium had shown similar results to the ones presented here, suggesting no uptake of cadmium through food but ascribing the differences in the metabolic rate of the daphnids (Smolders et al. 2005).

According to Chowdhury et al. (2008), and similarly to organisms like cyanobacteria, algae, and aquatic plants, low concentrations of nickel can be essential to crustaceans, molluscs, and fish, increasing their fitness. Hence, daphnids in an environment with lower food concentrations need to maintain their metabolic rates, and low nickel concentrations might be considered fundamental for their energy reserves.

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Chapter IV

Discussion and Conclusion

Generally, the impact of pesticides and metals in the aquatic environment is dependent on several characteristics, such as chemical and physical properties of the water or even the existence of several others chemical stressors. Studies keep showing that when a pesticide or a metal is found in the environment, co-existence with other chemicals (Barata et al. 2006; Xie et al. 2007), like other pesticides (Deneer 2000; Belden et al. 2007) or metals (Barata et al. 1998; Komjarova and Blust 2008; Vandenbrouck et al. 2009), or in different environmental conditions, as ranges of temperature, pH, hardness or food availability (Schubauer-Berigan et al. 1993; Hoang et al. 2004), the toxicity of the chemicals and the way they interact with the environment and living forms changes.

Several studies have been conducted worldwide in order to assess the behavior and toxicity of those chemicals alone or in interaction with others and the effects they may cause to the aquatic and terrestrial environment. For instance, in order to assess the toxicity of one certain chemical, studies with different organisms have been carried out, e.g. imidacloprid toxicity has been evaluated in the isopod *Porcellio scaber* (Drobne et al. 2008) and in the cladoceran *Daphnia magna* (Jemec et al. 2007) and nickel toxicity has been assessed in the worm *Tubifex tubifex* and in the cladoceran *Daphnia magna* (Fargasova 1994). Studies in order to predict the effects and evaluate the toxicity of chemicals interacting with others, e.g. mixtures with the same or different types of chemicals in the same or different organisms, have helped to understand the way a mixture may behave in the environment. For instance, Altenburger (2000) has tested sixteen similarly acting chemicals to the bacteria *Vibrio fischeri* and Gomez-Eyles et al. (2009) has tested the same chemical mixture in two different species, the the earthworm *Eisenia fetida* and the nematode *Caenorhabditis elegans*.

The overall results of this study showed that the neonicotinoids insecticides imidacloprid and thiacloprid caused effects in the reproduction and body length of the cladoceran *Daphnia magna*, within a range of concentrations tested. Following the same example, the heavy metal nickel, appeared to be highly toxic to the organism in concentrations higher than the EC_{50} equal to 289.29 μg/L. However, the insecticides are usually found in the environment in concentrations lower than the ones tested in this study, therefore do not cause irreversible effects to *D. magna.* In contrast to the heavy metal, nickel causes effects due to its presence in the environment in concentrations reported as the LOEC found in this study (LOEC = 250 μg/L) (Pane et al. 2003; Chowdhury et al. 2008a; Chowdhury et al. 2008b; Kozlova et al. 2009).

The final results of the single exposures presented in this study with the two neonicotinoid insecticides appeared to be in accordance with previous studies when it comes to imidacloprid (Young and Blakemore 1990; Jemec et al. 2007). For thiacloprid, as a relatively new insecticide, there has only been one study on the same organism yet in a different clone evaluating the effects on reproduction and growth and reporting EC_{50} values equal to 4.74 mg/L of the pesticide (Beketov and Liess 2008), a value approximately two times higher than the one obtained in the present study. This value was obtained from a 30 days post – exposure of *D. magna* to thiacloprid with the organism being exposed for the first 24 hours to different concentrations of the insecticide.

The metal showed that at concentrations lower than 150 µg/L can be considered as essential to aquatic organisms and cause no adverse effects to the tested organisms (Muyssen et al. 2004; Chowdhury et al. 2008b). Additionally, it was described by Faria et al. (2008) that levels of 450 µg Ni/L were observed in a aquatic system from a Portuguese mine (Faria et al. 2008). With this concentration daphnids are not able to breed properly and will die in some days (data not shown).

Several studies have been conducted in order to assess the toxicity of not just one chemical but a mixture of one or more (e.g. Enserink et al. 1991; Jonker et al. 2004; Cedergreen and Streibig 2005; De Zwart and Posthuma 2005; Cedergreen et al. 2007; Xie et al. 2007; Gomez-Eyles et al. 2009; Martin et al. 2009) and the environmental impact they might have. Using several models that can predict the toxicity and having them as a powerful tool (De Schamphelaere and Janssen 2002; Niyogi and Wood 2004; Jonker et al. 2005; Rinke and Vijverberg 2005; Baas et al. 2007; Cedergreen et al. 2008; Deleebeeck et al. 2008), scientists are trying to evaluate and predict the impact of chemicals in the environment, using either scenarios that match the real ones or worst case scenarios that sometimes do not apply to the reality.

It can be predicted that when an environmental parameter undergo a change in the environment at the same time a chemical is present, like a pesticide or a metal , the toxicity of that chemical to the organisms will be altered (Hanazato 2001; Pereira and Gonçalves 2007). Therefore a higher impact level will be observed in contrast to an environment exposed to the same chemicals under normal environmental conditions. The results presented in the current study appear to agree with this because different levels of food concentration affected the reproduction and body length of the cladoceran *Daphnia magna*. Several studies have used the same organism in order to assess the toxicity of different chemicals, like lindane (Antunes et al. 2004), or a different organism with a different chemical, like cadmium tested in *Echinisca triserialis*, (Chandini 1988) or in *Daphnia carinata* (Chandini 1989), both cladocerans, in different food concentrations and the results correspond to the fact that the toxicity of the chemicals change. Other studies show different environmental parameters acting as a natural stressor to organisms, like dissolved oxygen (Ferreira et al. 2008) or temperature (Martins et al. 2004; Tsui and Wang 2004; Osterauer and Köhler 2008) either alone or interacting with chemicals.

In order to interpret the effects of those combined stressors it is still considered a difficult task due to the lack of data on the evaluation of the chemical toxicity of environmental and other stressors for comparison and it is not always easy to compare results between mixtures in order to conclude which would be most probable to provoke negative effects to the environment and subsequently to the organisms (Ferreira et al. 2008).

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