Universidade de Aveiro Departamento de Biologia
2007

Abel Luis Gonçalves Ferreira

Efeito da combinação de stressores químicos e naturais em *Daphnia magna*

Universidade de Aveiro Departamento de Biologia
2007

Abel Luis Gonçalves Ferreira

Efeito da combinação de stressores químicos e naturais em *Daphnia magna*

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 Prof. Doutor Amadeu Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro

o júri

presidente

Amadeu Mortágua Velho da Maia Soares
Professor Catedrático do Departamento de Biologia da Universidade de Aveiro

Lúcia Maria Candeias Guilhermino
Professora Associada com agregação, ICBAS, Universidade do Porto

Susana Patrícia Mendes Loureiro
Investigadora, CESAM, Universidade de Aveiro

agradecimentos

Geralmente os agradecimentos são endereçados de acordo com o impacto directo ou indirecto de pessoas ou instituições, na execução de todo o trabalho conducente à elaboração desta dissertação. Nesse aspecto, não vou ser diferente…

Assim gostaria de começar pelas pessoas mais importantes… sim, aqui os primeiros serão sempre os primeiros! E não se esqueçam, são só palavras…

À minha esposa, Susana, pelo apoio, compreensão, ajuda no laboratório, mas sobretudo, pela paciência. Sei que não foi fácil, mas lembra-te… nos bons e maus momentos, na alegria e na tristeza, na saúde e na doença… blá, blá, blá…em todos os dias da nossa vida. Assim espero! Amo-te.

À minha mãe e avó, por tudo o que fizeram n estes 31 anos, mas sobretudo pela educação e estrutura moral que hoje me orgulho de possuir e da qual nunca abdiquei e tudo farei para nunca abdicar. Obrigado.

Ao Hugo e Raquel, pela força que me deram e pela amizade demonstrada desde os tempos da licenciatura. Sei que a distância física não enfraquece uma amizade tão segura e sólida como a nossa. È bom saber que posso contar convosco, para se rirem das minhas "brilhantes" chalaças!!!! Vocês acreditam que nem todos, têm esta opinião das minhas piadas?

Aos meus colegas e às minhas colegas de laboratório, e são tantos(as), pela vossa amizade, mas também pela ajuda nas funções que já desempenhei no laboratório. Vou fazer uma especial referência aos "fundadores", Pestana, Raquel, Joaquim, Filipa, Susana, Mónica, Bruno, Sara e Catarina.

À Daniela pela boa disposição e sorriso que inunda sempre que a vejo, o meu pobre e por vezes cansado e triste, espírito.

Ao Prof. Amadeu Soares, pela orientação mas em especial, às oportunidades que me deu e tem dado, de poder trabalhar ao longo destes 7 anos (desde Coimbra) e sobretudo de aprender e crescer profissionalmente. Obrigado pela confiança e apoio sempre que necessário e possível.

À Susana Loureiro (vénia), colega e espero, amiga, por tudo!

Um muito especial agradecimento, a uma pessoa que embora não esteja fisicamente presente, me marcou muito e me faz sentir orgulhoso pelo pai que tive ou melhor que ainda e sempre terei… Só, gostaria de ser 1/10 do pai e pessoa que foste, com todas as virtudes e defeitos que fazem do ser humano um ser imperfeito.

Por último, e também importante, a todas as dáfnias e algas sacrificadas em prol deste trabalho cientifico. O meu "muito obrigado" a vocês. Ah pois!

palavras-chave

Toxicidade de misturas, Adição de Concentração, Acção independente, *Daphnia magna*, stressores naturais, metais, pesticidas.

resumo

A contaminação ambiental é quase sempre caracterizada pela combinação de factores de stress de várias origens (biológica, química e física).

Na agricultura, a grande diversidade de colheitas e das pestes que as atingem, conduz à utilização de diversos tipos de tratamentos e à aplicação de uma larga diversidade de produtos químicos. O fungicida carbendazim, pertencente ao grupo dos benzimidazóis, e é um dos mais utilizados em todo o mundo, enquanto que o insecticida organofosforado clorpirifos, também globalmente utilizado, é somente o mais vendido em Portugal.

Igualmente, da actividade industrial advêm alguns dos mais frequentes contaminantes encontrados nos nossos ecossistemas. Os metais pesados, como o cádmio e o níquel, resultantes de actividades mineiras e metalúrgicas, são importantes agentes de toxicidade, uma vez que induzem vários tipos de efeitos nocivos.

O fenómeno das alterações climáticas, que já se faz sentir e que irá intensificar-se nas próximas décadas, levando ao surgimento de condições ambientais extremas durante longos períodos de tempo, será também uma nova fonte de agentes stressores que importa ter em consideração.

A Avaliação do Risco Ecológico (ARE) tem como objectivo avaliar a probabilidade de determinados efeitos ecológicos adversos ocorrerem como resultado da exposição a um ou mais stressores (U.S.EPA 1992). Uma nova abordagem baseada na avaliação dos efeitos cumulativos da toxicidade resultante da acção de múltiplos stressores surgiu nos últimos anos, levando ao desenvolvimento de novas ferramentas para descrever e analisar os efeitos das misturas.

Os modelos teóricos utilizados na avaliação da toxicidade de misturas são baseados nos conceitos de adição de concentração (AC) e acção independente (AI) e têm sido recentemente aplicados por diversos autores.

No entanto, têm vindo a ser observados desvios a estes dois modelos conceptuais, principalmente devido a interacções que podem ocorrer ao nível toxicocinético ou toxicodinâmico e produzir padrões de resposta diferentes; sinergismo onde os efeitos são mais severos; antagonismo com efeitos menos severos; dependentes do nível da dose ou do rácio entre os dois componentes da mistura.

A avaliação dos efeitos dos contaminantes em organismos aquáticos inclui geralmente a avaliação de parâmetros agudos (mortalidade) e crónicos, como a reprodução, o crescimento e a actividade alimentar. O cladócero, Daphnia magna tem sido utilizado, durante muitos anos, em ensaios de toxicidade devido à sua elevada sensibilidade, fácil manuseamento e manutenção em cultura e ainda pela sua alta taxa reprodutiva.

Este trabalho tem como principais objectivos: avaliar a toxicidade de misturas heterogéneas, compostas por metais (Cd e Ni) e pesticidas com modos de acção ambíguos e diferente natureza química (carbendazim e clorpirifos); avaliar a toxicidade resultante da introdução de stressores naturais (temperatura e oxigénio dissolvido) em combinação com os diferentes químicos; obter padrões de comportamento das misturas em função dos conceitos de adição de concentração (AC) e acção independente (AI) e verificar a ocorrência de desvios (sinergismo, antagonismo e dependência da dose ou do rácio da mistura); verificar a existência de sinergismos associados às condições extremas de temperaturas altas e de hipoxia e avaliar a adequação dos modelos AI e AC na interpretação do comportamento da toxicidade conjunta de stressores com modos de acção diferentes ou semelhantes.

Este estudo é um excelente exemplo da diversidade de efeitos e comportamentos que podem resultar da combinação de tóxicos bastante comuns no nosso ambiente, uma vez que todos os possíveis desvios aos modelos ocorreram (sinergismo, antagonismo, dependência da dose ou do rácio da mistura).

Verificaram-se alterações de efeitos sinergísticos para efeitos antagonísticos, respectivamente, de exposições letais para subletais, em praticamente todas as misturas ajustadas ao modelo de acção independente.

Os efeitos sinergísticos normalmente associados a condições ambientais extremas como altas temperaturas e baixas concentrações de oxigénio dissolvido foram observados nas exposições agudas, a partir de ajustes ao modelo de acção independente.

O modelo matemático aqui utilizado e mostrou ser no geral, uma boa ferramenta na avaliação de respostas dadas pela D. magna exposta a misturas binárias.

A validação a partir de estudos toxicocinéticos e toxicodinâmicos parece ser de primordial importância para este tipo de avaliações, uma vez que permitirá compreender todos os processos fisiológicos envolvidos na complexidade das misturas.

keywords

Mixtures toxicity, concentration addition, independent action, *Daphnia magna,* natural stressors, metals and pesticides

abstract

Environmental contamination is often characterised by the combination of stress factors of various sources (biological, chemical and physical).

In agriculture, the diversity of crops and respective pests lead to the use of a large variety of treatments and to the application of a wide miscellaneous of chemical compounds. Carbendazim, a benzimidazole fungicide, is one of the most used pesticides in all world, while chlorpyrifos, an organophosphorus insecticide, also widely used is the most sold insecticide in Portugal.

Also, from the industrial activity, many compounds that are manufactured can be usually found in our ecosystems. Heavy metals, such as cadmium and nickel, resulting from metallurgic and mining activities, are one of those chemicals and are considered important toxic agents for inducing several types of harmful effects.

Climate changes phenomenon can increase adverse effects during the next decades, leading to the emergence of extreme environmental conditions during long time periods, and will also be a source of stressors that should be taken into consideration.

Ecological risk assessment (ERA) has as main objective to assess the likelihood of occurrence of adverse ecological effects as a result of exposure to single or multiple stressors. A new approach based on the evaluation of cumulative effects resulting from the toxicity of the multiple stressors action emerged in the last few years, conducting to the development of novel tools to describe and analyse mixtures effects.

Theoretical models based on the two widely concepts used on toxicity of mixtures, concentration addition (CA) and independent action (IA), have been recently applied by several authors.

Deviations from these two conceptual models have also been observed, probably due to interactions that may occur at toxicokinetics or toxicodynamics levels and produce different behaviour patterns, according to a more severe effect (synergism), less severe effect (antagonism), dose level or dose ratio dependent. An evaluation of the contaminants effects in aquatic organisms usually includes assessment of acute and sublethal parameters such as mortality and feeding activity, respectively.

Daphnia magna has been used during many years in standard toxicity tests due to its high sensitivity, easy handling and high reproductive rate.

The main objectives of this study are: to evaluate the toxicity of heterogeneous mixtures of metals (Cd and Ni) and pesticides with ambiguous modes of action and different chemical nature (carbendazim and chlorpyrifos); to evaluate the toxicity of natural stressors (temperature and dissolved oxygen) in combination with the different chemicals; to obtain behaviour patterns as a function of CA

and IA concepts and verify the occurrence of possible deviations (synergism, antagonism, dose level dependent and dose ratio dependent); to check the occurrence of synergisms associated to extreme conditions of high temperatures and hypoxia and to evaluate the adequacy of the IA and CA models on the interpretation of the joint toxicity behaviour of chemicals with dissimilar or similar modes of action.

This work comprises a diversity of effects and behaviours that can result from the combination of very common toxicants once all the possible deviations have occurred (synergism, antagonism, dose level and dose ratio dependency).

A switch from synergistic to antagonistic effects has occurred from lethal to sublethal exposures, in practically all the mixtures fitted to IA model.

Synergistic effects normally associated to extreme environmental conditions such as high temperatures and low dissolved oxygen levels were observed on acute exposures, after the fit to IA model.

The mathematical model used in this work showed to be, in general, a good tool to evaluate the responses given by the exposure of D. magna to binary mixtures.

Validation from toxicokinetics and toxicodynamics studies seems to be of crucial importance for this kind of approach, once it permits the understanding of toxicological pathways involved on complex mixtures.

"Coloquei-te no centro do mundo, para que melhor pudesses contemplar o que o mundo contém. Não te fiz nem celeste nem terrestre, nem mortal nem imortal, para que tu, livremente, tal como um bom pintor ou um hábil escultor, dês acabamento à forma que te é própria."

PICO DE MIRÂNDOLA
Oratio de Hominis Dignitate

Índice

Lista de Figuras

II. Fig.8 – Dose-response data from 24-hrs exposure of *D. magna* to the combinations Ni+HT (upper row) and Ni+LT (lower row) showing synergistic and a dose ratio patterns. Representations of feeding responses: a) 3D mesh and b) 2D isobolic surfaces. Control value for temperature is 20ºC.

II. Fig.9 – Dose-response data from 4-hrs post-exposure period of *Daphnia magna* to combined Ni+HT (upper row) and Ni+LT (lower row), illustrating a synergistic deviation and no deviation from IA model, respectively. Representations of feeding responses: a) 3D mesh and b) 2D isobolic surfaces. Control value for temperature is 20ºC.

II. Fig.10 – Dose-response data from 24-hrs exposure period of *Daphnia magna* to combined CPF+HT (upper row) and CPF+LT (lower row), illustrating a no deviation and a dose ratio deviation from IA model, respectively. Representations of feeding responses: a) 3D mesh and b) 2D isobolic surfaces. Control value for temperature is 20ºC.

II. Fig.11 – Dose-response data from 4-hrs post-exposure period of *Daphnia magna* to combined CPF+LT, illustrating a no deviation from IA model. Representations of feeding responses: a) 3D mesh and b) 2D isobolic surfaces. Control value for temperature is 20ºC.

III. Fig.1 – A schematic design of toxic units combinations used for a) chemical vs. natural stressor combination – factorial design and b) chemical vs. chemical mixtures – ray design.

III. Fig.2 – 24 hours exposure and 4-hrs post- exposure feeding rates (Sd. error bars) on *D. magna* at different concentrations of cadmium (a), carbendazim (b) and dissolved oxygen (c). Control value for D.O of 9mg/L, is represented by a •••••• dotted line for 24-hrs exposure and a ---- dashed line for 4-hrs post-exposure data. * - significant differences at $p<0.05$.

III. Fig.3 – Dose-response relationship for the binary mixture Cd+CBZ showing a dose ratio deviation from the IA model for *D. magna*'s survival: a) 3D mesh and b) 2D isobolic surfaces.

III. Fig.4 – Dose-response relationship for the combination of Cd+D.O. showing a synergistic deviation from the IA model (upper row) and an antagonistic deviation from the CA model (lower row) for *D. magna*'s survival: a) 3D mesh and b) 2D isobolic surfaces. Control value for D.O is 9mg/L.

III. Fig.5 – Dose-response relationship for the combination of CBZ+D.O. showing a synergistic deviation from the IA for *D. magna*'s survival: a) 3D mesh and b) 2D isobolic surfaces. Control value for D.O is 9mg/L.

III. Fig.6 – Dose-response data from 24-hrs exposure for the binary mixture CBZ+Cd, showing an antagonistic deviation from the IA model for *Daphnia magna*'s feeding response: a) 3D mesh and b) 2D isobolic surfaces.

III. Fig.7 – Dose-response data from 24-hrs exposure for the combination of Cd+D.O., showing an antagonistic deviation from the CA model (upper row) and the IA model (lower row) for *Daphnia magna*'s feeding response: a) 3D mesh and b) 2D isobolic surfaces. Control value for D.O is 9mg/L.

III. Fig.8 – Dose-response data from 4-hrs post-exposure for the combination of Cd + D.O, showing an antagonistic deviation from the CA model for *Daphnia magna*'s feeding response: a) 3D mesh and b) 2D isobolic surfaces. Control value for D.O is 9mg/L.

III. Fig.9 – Dose-response data from 24-hrs exposure for the combination of CBZ + D.O, showing an antagonistic deviation from the IA model: for Daphnia magna's feeding response: a) 3D mesh and b) 2D isobolic surfaces. Control value for D.O is 9mg/L.

Lista de Tabelas

III. Table 3 – 48-hrs LC50 values for cadmium and carbendazim at low dissolved oxygen levels (D.O). Data obtained from acute tests on *D. magna*. Control value for D.O was 9mg/L.

III. Table 4 – 24-hrs EC50 and 4-hrs EC50 values for exposure and post-exposure, respectively, of cadmium and carbendazim, at low dissolved oxygen levels (D.O). Data obtained from feeding tests on *D. magna*. Control value for D.O was 9mg/L.

Capítulo I

Introdução geral e objectivos

I. Introdução Geral e Objectivos

I.1. *Introdução*

A contaminação ambiental é, nos tempos modernos, caracterizada por uma grande diversidade de contaminantes, resultantes essencialmente da actividade humana.

Uma das actividades humanas que mais impacto tem sobre os ecossistemas é a agricultura. A aplicação de pesticidas em grandes áreas é normalmente feita por pulverização aérea, levando à contaminação de áreas não afectas a este tipo de actividade. Mesmo quando feita em menor escala, a contaminação de outros locais acaba por ser inevitável devido a fenómenos como, a volatilização para a atmosfera, as escorrências ou a contaminação de águas subterrâneas por infiltração. A grande diversidade de colheitas e das pestes que as atingem, conduz à utilização de diversos tipos de tratamentos e à aplicação de uma ampla variedade de produtos químicos.

O fungicida carbendazim, pertencente ao grupo dos benzimidazóis, é um dos mais utilizados em todo o mundo. É um fungicida sistémico que além de afectar a divisão nuclear no momento da separação dos cromossomas efectuada pelo fuso mitótico (European Medicines Agency 2004), é também um inibidor da actividade da enzima acetilcolinesterase (AChE) (Cuppen *et al*. 2000) resultando na acumulação do neurotransmissor acetilcolina (ACh) no receptor pós-sináptico e na consequente sobrestimulação do sistema nervoso periférico dos organismos (Printes and Callaghan 2004).

O insecticida organofosforado, clorpirifos, é um dos mais utilizados em todo o mundo e em Portugal chegou às 100 toneladas de produto vendido em 2003 (Vieira 2005). A sua rápida degradação no meio ambiente e aparente não-acumulação ao longo da cadeia trófica, são características que podem explicar parte do seu sucesso. No entanto, o seu efeito tóxico não se limita apenas às espécies alvo, mas atinge também outros organismos tais como peixes (Pathiratne 2006) e invertebrados aquáticos (Cuppen *et al*. 2002). O principal mecanismo de acção tóxica do clorpirifos situa-se ao nível do sistema nervoso

central e periférico, onde inibe a actividade da enzima acetilcolinesterase (AChE) (Fulton and Key 2001). Esta enzima é a responsável pela hidrólise do neurotransmissor acetilcolina (ACh), e consequente estimulação dos receptores colinérgicos. O processo de inibição da AChE pelo clorpirifos é irreversível estando a recuperação dependente da produção de nova enzima. A sua inibição conduz à acumulação de ACh, e na consequente sobrestimulação do sistema nervoso autónomo e motor, responsáveis pelo controlo de funções relacionadas com o sistema respiratório, cardíaco, digestivo e locomoção. A extensão dos efeitos nocivos resultantes da acumulação de ACh, depende da distribuição do tóxico pelo organismo e também do tipo de receptores colinérgicos envolvidos (Mileson *et al.* 1998). Recentemente, a acção deste insecticida foi também associada à formação de espécies reactivas de oxigénio indutoras de stress oxidativo (Jager *et al*. 2007).

A crescente industrialização verificada nas últimas décadas, conduziu inevitavelmente à produção de grandes quantidades de resíduos, acabando a maioria destes por criar impactos negativos nos ambientes terrestres e aquáticos. Os metais pesados, como o cádmio e o níquel, resultantes de actividades mineiras e metalúrgicas, são importantes agentes de toxicidade uma vez que induzem vários tipos de efeitos nocivos. O cádmio e o níquel, para além de potenciarem a produção de espécies reactivas de oxigénio (stress oxidativo) (Chen *et al*. 2003; Pinto *et al*. 2003), podem também causar danos no ADN celular (Badisa *et al.* 2007; Lynn *et al.* 1997), assim como a despolarização das membranas e acidificação do citoplasma das células (Conner and Schimid 2003).

O fenómeno das alterações climáticas, que aliás, já se faz sentir e que provavelmente será intensificado nas próximas décadas, levando ao surgimento de condições ambientais extremas durante longos períodos de tempo, será uma nova fonte de agentes stressores que importa ter em consideração (Intergovernmental Panel on Climate Change - IPCC. 2001). Assim, a temperatura será, no futuro, um dos factores de maior importância uma vez que afecta directamente os organismos vivos por alteração do metabolismo e dos níveis de oxigénio (Viswanathan and Murti 1989) e de forma indirecta, ao facilitar o processo de difusão dos compostos tóxicos no organismo. A alteração dos níveis

de oxigénio terá impactos severos nas comunidades aquáticas nomeadamente por perdas na diversidade (Connolly *et al*. 2004). Os baixos níveis de oxigénio dissolvido (O.D) acarretam ainda efeitos negativos em alguns processos fisiológicos, especialmente ao nível do sistema circulatório (Paul *et al.* 1997) e no metabolismo (Wiggins and Frappell 2000). Em resposta aos baixos níveis de O.D, alguns organismos têm a capacidade de aumentar a produção de hemoglobina que, tal como referido anteriormente, pode levar ao aparecimento de stress oxidativo (Gorr *et al.* 2004).

A ARE tem como objectivo avaliar a probabilidade de determinados efeitos ecológicos adversos ocorrerem como resultado da exposição a um ou mais stressores (U.S.EPA 1992). Durante décadas a ARE focalizou toda a sua atenção na acção de químicos em ambientes relativamente homogéneos e na aplicação de regras simples de forma a estimar a toxicidade de um composto tóxico a um determinado receptor/organismo (Mckone and Ryan 1989). No entanto numa abordagem cumulativa, estes receptores não estão sujeitos a um stressor isolado, e muito menos, num ambiente de condições constantes (homogéneo), mas sim a misturas de químicos e outros stressores naturais (por exemplo, seca severa, baixos níveis de oxigénio e temperaturas extremas) em ambientes heterogéneos (Heugens *et al*. 2001). Esta combinação de stressores pode resultar em efeitos conjuntos mais ou menos severos do que aqueles esperados, tendo em conta a toxicidade individual de cada um. Esta nova realidade levou a USEPA[1] e outras entidades reguladoras a alargarem a caracterização de risco ecológico às misturas, de forma a tornarem as avaliações de risco ecologicamente relevantes (European Commission, 1996; USEPA, 1999).

Os modelos teóricos baseados em dois conceitos utilizados na toxicidade de misturas, adição de concentração (AC) e acção independente (AI) têm sido recentemente aplicados por diversos autores (Altenburger *et al*. 2000; Backhaus *et al.* 2000; Barata *et al*. 2006; Cassee *et al*. 1998; Jonker *et al*. 2004; Jonker *et al*. 2005).

O conceito de acção independente (AI) assume que os stressores em causa produzem os seus efeitos através de diferentes modos de acção sem

[1] - abreviatura para "United States Environmental Protection Agency"

interacção mútua (Olmstead and LeBlanc 2005) e é descrita pela fórmula matemática baseada em probabilidades:

$$Y = u_{\max} \prod_{i=1}^{n} q_i(c_i)$$

onde, Y corresponde à resposta biológica, c_i é a concentração do tóxico *i* na mistura, $q_i(c_i)$ a probabilidade da resposta não ocorrer, u_{max} o valor da resposta no controlo ou máxima para o parâmetro em estudo e ∏ a função de multiplicação (Jonker *et al.* 2005).

O conceito de adição de concentração (AC) é baseado no mesmo modo de acção (MoA) dos stressores em mistura (Altenburger *et al.* 2000) e assume que a toxicidade relativa desta é idêntica à toxicidade individual de cada um dos stressores, matematicamente expressa como:

$$\sum_{n}^{i=1} \frac{C_i}{ECx_i} = 1$$

sendo, C_i a concentração do tóxico *i* na mistura e ECx_i a concentração de tóxico relativa a *x*% do efeito da mistura.

Estes dois modelos teóricos utilizam o conceito de unidade tóxica (UT) definido como a contribuição relativa de uma determinada concentração de um tóxico na produção de x % de efeito no organismo causado por esse mesmo tóxico isoladamente (Norwood *et al.* 2003), isto é, se por exemplo o valor de LC_{50} de um determinado composto Y for 0.5mg/L, a uma concentração de 0.5mg$_Y$/L corresponderá 1UT e às concentrações de 1mg$_Y$/L e 0.25mg$_Y$/L corresponderão 2UT e 0.5UT, respectivamente.

A aplicação dos modelos de toxicidade, aos conceitos de CA e AI, tendo em conta modos de acção (MoA) similares ou diferentes, respectivamente, nem sempre é linear. Muitas vezes, a informação existente sobre o MoA dos vários elementos que compõem uma determinada mistura, é escassa ou ambígua. Em certas misturas, os vários componentes podem actuar sobre o mesmo receptor ou alvo celular, mas no entanto produzirem efeitos adversos em processos fisiológicos diferentes, ou seja, enquanto que ao nível celular, o comportamento

pode ser associado ao conceito de adição de concentração, já o facto de induzirem diferentes mecanismos, pode ser interpretado como seguindo o modelo de acção independente. Outras podem, por exemplo, ser compostas por elementos que actuem num mesmo órgão mas em diferentes receptores celulares. Assim e para uma correcta avaliação dos efeitos resultantes de misturas complexas, torna-se necessário obter informação detalhada sobre a composição da mistura, nível de exposição, mecanismo de acção e respectivos receptores de cada um dos componentes (Cassee *et al*. 1998).

Existem também compostos que podem interagir uns com os outros modificando a magnitude e por vezes a natureza dos efeitos tóxicos. As interacções podem ocorrer ao nível toxicocinético (nos processos de absorção, distribuição, metabolismo e excreção) ou toxicodinâmico (na ligação aos receptores e alvos celulares) (Cassee *et al.* 1998) e produzir padrões de resposta diferentes consoante os efeitos são mais severos (sinergismo), menos severos (antagonismo), dependentes do nível da dose ou do rácio entre os dois componentes da mistura (Jonker *et al.* 2005).

A avaliação dos efeitos dos contaminantes em organismos aquáticos usualmente inclui a avaliação de parâmetros agudos (mortalidade) e crónicos, como parâmetros reprodutores, de crescimento ou actividade alimentar. Os dafnídeos, especialmente a *Daphnia magna*, têm sido utilizados durante muitos anos em ensaios de toxicidade devido à sua elevada sensibilidade, fácil manuseamento e alta taxa reprodutora (Munzinger and Monicelli 1992). O facto de se reproduzirem assexuadamente (por partenogénese) em condições favoráveis ao seu desenvolvimento e portanto em laboratório, permite diminuir as componentes de variação genética e ambiental dos ensaios laboratoriais (Barata et al. 2000). A exposição a baixas concentrações de compostos tóxicos afecta, geralmente, o comportamento alimentar, a reprodução ou o crescimento da *Daphnia magna* como foi já descrito por vários autores (Barata *et al*. 2006; De Schamphelaere *et al.* 2007; Dzialowski *et al*. 2006; McWilliam and Baird 2002; Muyssen *et al*. 2006).

I.2. *Objectivos e estrutura de tese*

Este trabalho pretende avaliar o tipo de respostas associadas à toxicidade conjunta resultante de misturas de vários químicos com modos de acção semelhantes e diferentes e ainda da combinação destes com alguns stressores naturais. Para esse efeito, foram testados dois pesticidas, o carbendazim e o clorpirifos, dois metais pesados, o níquel e o cádmio e dois stressores ambientais, temperatura e oxigénio dissolvido no cladócero *Daphnia magna* Straus de forma a avaliar a toxicidade combinada (toxicidades aguda e crónica), em termos de comportamento das misturas. Foi utilizado um modelo matemático, baseado nos conceitos de adição de concentração e acção independente e determinada a existência de possíveis desvios resultantes da interacção dos componentes em mistura (sinergismo, antagonismo, dependência da dose ou rácio da mistura).

Assim, os objectivos gerais deste trabalho foram:

- Avaliar a toxicidade de misturas heterogéneas, compostas por um metal e um pesticida, tendo em conta os seus modos de acção, sejam eles diferentes ou ambíguos;

- Avaliar a toxicidade resultante da introdução de stressores naturais em combinação com os diferentes químicos e verificar a existência de efeitos sinergísticos;

- Verificar a ocorrência de diferenças no comportamento das misturas, em termos de toxicidade aguda e crónica no cladócero *D. magna*;

- Avaliar a robustez do modelo matemático utilizado na previsão da toxicidade das misturas binárias.

De forma a atingir os objectivos gerais acima propostos, a tese foi organizada da seguinte forma:

- Um primeiro capítulo, correspondendo à introdução geral onde foi feito o enquadramento deste estudo no âmbito da actual avaliação de risco ecológico (ARE), a introdução dos conceitos de modelos de toxicidade de misturas (adição de concentração e acção independente) e respectivos desvios, uma breve

caracterização e enquadramento dos stressores químicos e naturais bem como do organismo teste, *D. magna*, utilizados neste trabalho. No final foram descritos os principais objectivos.

- Um segundo capítulo intitulado **"Joint toxicity assessment of nickel and chlorpyrifos on life parameters of *D. magna*: influence of temperature on chemicals toxicity"**, onde se pretendeu determinar o tipo de resposta, em termos de toxicidade conjunta, associado a misturas de compostos químicos com diferentes modos de acção (níquel e chlorpirifos), bem como, a combinações com diferentes temperaturas. Os objectivos específicos deste primeiro capítulo, consistem em:

- Avaliar a toxicidade (aguda e crónica) de misturas heterogéneas, compostas por um metal (níquel) e um insecticida (clorpirifos), substâncias com diferentes modos de acção e a sua adequação ao modelo de acção independente;

- Avaliar a toxicidade resultante da introdução de um stressor natural (temperatura) em combinação com os diferentes químicos, e verificar a existência de sinergismos;

- Obter padrões de comportamento das misturas em função do conceito de acção independente (AI) e verificar a ocorrência de desvios (sinergismo, antagonismo e dependência da dose ou rácio da mistura).

- Um terceiro capítulo intitulado "**Prediction of mixtures toxicity on survival and feeding parameters of *Daphnia magna*: Evaluation of binary combinations of cadmium, carbendazim and low dissolved oxygen**", que pretende avaliar o tipo de resposta, em termos de toxicidade conjunta, associado a misturas de stressores com modos de acção diferentes e outros com modos de acção ambígua, isto é, que podem induzir uma mesma resposta fisiológica, mas através de diferentes mecanismos. Ajustando as respostas aos dois modelos de toxicidade de misturas, acção independente e adição de concentração, e verificando a existência de desvios, poderemos determinar de que forma a toxicidade resultante pode ser interpretada.

Deste modo, os objectivos específicos deste terceiro capítulo, consistem em:

- Avaliar a toxicidade conjunta (aguda e crónica), de misturas entre o cádmio e o carbendazim, substâncias com diferentes modos de acção e a sua adequação ao modelo de acção independente;

- Avaliar a toxicidade resultante da introdução do oxigénio dissolvido, como stressor natural, em combinação com o cádmio e com o carbendazim e verificar a existência de sinergismos;

- Obter padrões de comportamento das misturas em função dos conceitos de adição de concentração (AC) e acção independente (AI) e verificar a ocorrência de desvios (sinergismo, antagonismo e dependência da dose ou rácio da mistura);

- Avaliar a adequação dos modelos AI e AC na interpretação do comportamento da toxicidade conjunta de químicos com modos de acção diferentes ou semelhantes.

- Um quarto e último capítulo, dedicado à discussão e às conclusões finais onde, para além da comparação dos resultados obtidos neste trabalho com alguns dos dados já publicados por outros autores, são descritas as principais conclusões dos capítulos II e III.

I.3. *Referências Bibliográficas*

Altenburger R, Backhaus T, Boedeker W, Faust M, Scholze M, Grimme LH. 2000. Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: Mixtures composed of similarly acting chemicals. Environmental Toxicology and Chemistry 19(9):2341-2347.

Backhaus T, Altenburger R, Boedeker W, Faust M, Scholze M, Grimme LH. 2000. Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to *Vibrio fischeri*. Environmental Toxicology and Chemistry 19(9):2348-2356.

Badisa VLD, Latinwo LMO, Caroline O., Ikediobi CO, Badisa RB, Ayuk-Takem LT, Nwoga J, West J. 2007. Mechanism of DNA damage by cadmium and interplay of antioxidant enzymes and agents. Environmental Toxicology 22(2):144-151.

Barata C, Baird D, Nogueira A, Soares A, Riva M. 2006. Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. Aquatic Toxicology 78(1):1-14.

Barata C, Baird DJ, Minarro A, Soares A. 2000. Do genotype responses always converge from lethal to non-lethal toxicant exposure levels? Hypothesis tested using clones of *Daphnia magna* Straus. Environmental Toxicology and Chemistry 19(9):2314-2322.

Cassee FR, Groten JP, van Bladeren PJ, Feron VJ. 1998. Toxicological evaluation and risk assessment of chemical mixtures. Critical Reviews in Toxicology 28(1):73-101.

Chen CY, Wang YF, Lin YH, Yen SF. 2003. Nickel-induced oxidative stress and effect of antioxidants in human lymphocytes. Archives of Toxicology 77(3):123-130.

Conner SD, Schimid SL. 2003. Regulated portals of entry into the cell. Nature 422:37-44.

Connolly NM, Crossland MR, Pearson RG. 2004. Effect of low dissolved oxygen on survival, emergence, and drift of tropical stream macroinvertebrates. Journal of the North American Benthological Society 23(2):251-270.

Cuppen JGM, Crum SJH, Heuvel HHVd, Smidt RA, Brink PJVd. 2002. Effects of a Mixture of Two Insecticides in Freshwater Microcosms: I. Fate of Chlorpyrifos and Lindane and Responses of Macroinvertebrates. Ecotoxicology 11(3):165-180.

De Schamphelaere KAC, Forrez I, Dierckens K, Sorgeloos P, Janssen CR. 2007. Chronic toxicity of dietary copper to *Daphnia magna*. Aquatic Toxicology 81(4):409-418.

Dzialowski EM, Turner PK, Brooks BW. 2006. Physiological and reproductive effects of beta adrenergic receptor antagonists in *Daphnia magna*. Archives of Environmental Contamination and Toxicology 50(4):503-510.

European Commission. 1996. Technical guidance document in support of Commission Directive 93/67/EEC on risk assessment for new notified substances and Commission Regulation (EC) Nº 1488/94 on risk assessment for existing substances. Office for Official Publications of the European Communities, Luxembourg.

European Medicines Agency. 2004. Fenbendazole (extrapolation to all ruminants) summary report (4). EMEA/MRL/866/03- Final Committee for Medicinal Products for Veterinary Use. Veterinary Medicines and Inspections, London, UK.

Gorr T, Cahn J, Yamagata H, Bunn H. 2004. Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna*. Is hypoxia-inducible factor-dependent. The Journal of Biological Chemistry 279(20):36038-36047.

Heugens EHW, Hendriks AJ, Dekker T, van Straalen NM, Admiraal W. 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Critical Reviews in Toxicology 31(3):247-284.

Intergovernmental Panel on Climate Change - IPCC. 2001. Climate change 2001: impacts, adaptation and vulnerability, Cambridge University Press, Cambridge 1042 p.

Jager T, Crommentuijn T, Van Gestel CAM, Kooijman SALM. 2007. Chronic exposure to chlorpyrifos reveals two modes of action in the springtail *Folsomia candida*. Environ. Pollut. 145:452-458.

Jonker MJ, Piskiewicz AM, Castella NII, Kammenga JE. 2004. Toxicity of binary mixtures of cadmium-copper and carbendazim-copper to the nematode *Caenorhabditis elegans*. Environmental Toxicology and Chemistry 23(6):1529-1537.

Jonker MJ, Svendsen C, Bedaux JJM, Bongers M, Kammenga JE. 2005. Significance testing of synergistic/antagonistic, dose-level dependent, or dose ratio-dependent effects in mixture dose response analysis. Environmental Toxicology and Chemistry 24(10):2701-2713.

Lynn S, Yew FH, Chen KS, Jan KY. 1997. Reactive oxygen species are involved in nickel inhibition of DNA repair. Environmental and Molecular Mutagenesis 29(2):208-216.

McWilliam RA, Baird DJ. 2002. Postexposure feeding depression: A new toxicity endpoint for use in laboratory studies with *Daphnia magna*. Environmental Toxicology and Chemistry 21(6):1198-1205.

Mileson BE, Chambers JE, Chen WL, Dettbarn W, Ehrich M, Eldefrawi AT, Gaylor DW, Hamernik K, Hodgson E, Karczmar AG and others. 1998. Common mechanism of toxicity: A case study of organophosphorus pesticides. Toxicological Sciences 41(1):8-20.

Munzinger A, Monicelli F. 1992. Heavy-Metal Cotolerance in a Chromium Tolerant Strain of *Daphnia magna*. Aquatic Toxicology 23(3-4):203-216.

Muyssen BTA, De Schamphelaere KAC, Janssen CR. 2006. Mechanisms of chronic waterborne Zn toxicity in *Daphnia magna*. Aquatic Toxicology 77(4):393-401.

Norwood WP, Borgmann U, Dixon DG, Wallace A. 2003. Effects of metal mixtures on aquatic biota: A review of observations and methods. Human and Ecological Risk Assessment 9(4):795-811.

Olmstead AW, LeBlanc GA. 2005. Toxicity Assessment of Environmentally Relevant Pollutant Mixtures Using a Heuristic Model. Integrated Environmental Assessment and Management 1(2):114-122.

Pathiratne LWHUCaA. 2006. Body size-related differences in the inhibition of brain acetylcholinesterase activity in juvenile *Nile tilapia* (*Oreochromis niloticus*) by chlorpyrifos and carbosulfan. Ecotoxicology and Environmental Safety.

Paul RJ, Colmorgen M, Hüller S, Tyroller F, Zinkler D. 1997. Circulation and respiratory control in millimetre-sized animals (*Daphnia magna, Folsomia candida*?) studied by optical methods. Journal of Comparative Physiology B 167(6):399-408.

Pinto E, Sigaud-Kutner TCS, Leitao MAS, Okamoto OK, Morse D, Colepicolo P. 2003. Heavy metal-induced oxidative stress in algae. Journal of Phycology 39(6):1008-1018.

USEPA 1999. Reregsitration eligibility science chapter for chlorpyrifos: Fate and environmental risk assessment chapter. Docket Control Number OPP-34203 (0030). Washington, DC.

Vieira MM. 2005. Venda de produtos fitofarmacêuticos em Portugal em 2003. PPA(AB)-01/05. Direcção-Geral de Protecção das Culturas, Oeiras. Ministério da Agricultura, do Desenvolvimento Rural e das Pescas.

Viswanathan PN, Murti CRK. 1989. Effects of temperature and humidity on ecotoxicology of chemicals - In : Bourdeau P. (ed.), Haines J.A. (ed.), Klein W. (ed.), Krishna Murti C.R. (ed.) Ecotoxicology and climate. New York : J. Wiley, . p. 139-154 p.

Wiggins PR, Frappell PB. 2000. The influence of haemoglobin on behavioural thermoregulation and oxygen consumption in *Daphnia carinata*. Physiological and Biochemical Zoology 73(2):153-160.

II. Joint toxicity assessment of nickel and chlorpyrifos on life parameters of *D. magna*: influence of temperature on chemicals toxicity

Abel L. G. Ferreira*, Susana Loureiro and Amadeu M. V. M. Soares
Department of Biology & CESAM, University of Aveiro
3810-193 Aveiro, Portugal
*email: aferreira@bio.ua.pt

Abstract – Environmental contamination is often characterised by a combination of stress factors of various sources (biological, physical and chemical). The predictability of their joint effects is an important stage on environmental risk assessment procedures. The conceptual model for mixtures evaluation based on dissimilar individual compounds MoA, independent action (IA) and deviations to synergism/antagonism, dose-ratio and dose-level dependency were used. The present study analysed the single and combined effects of nickel (metal), chlorpyrifos (insecticide) and low/high temperatures (natural stressor) on life-cycle parameters (survival and feeding) of the water flea *Daphnia magna* Straus. The results of chemical single exposures revealed an increase of toxicity as concentrations of nickel and chlorpyrifos increases. For acute exposures to temperature, a 50% of mortality was obtained at 30.7ºC and 4.2ºC while the same level of reduction on feeding activity was observed at 22.6ºC and 16.01ºC.

Chlorpyrifos induced the more severe effects at the lethal level, since synergism was observed in all combinations with low/high temperatures. A similar conclusion can be taken for nickel at sublethal levels of exposure, where, again, synergistic effects resulted from all the combinations that included this divalent metal. Also, on mixtures between nickel and chlorpyrifos, synergism was observed, in both lethal and sublethal experiments. The synergistic effects usually associated with extreme environmental conditions, such as high temperatures, by directly and/or indirectly increasing the toxicity of chemicals were observed in this study, except on the combination between high temperatures and chlorpyrifos at sublethal levels of exposure. Validation from toxicokinetics and toxicodynamics modelling studies

should be made in the future to understand the toxicological pathways involved on complex combinations of stressors. We can conclude that the nested model used here showed to be in general a good tool to evaluate the responses given by daphnids exposed to binary mixtures.

Keywords – Independent action; Synergism/antagonism; dose-ratio dependence; Nickel; Chlorpyrifos; Temperature; *Daphnia magna*; Mixture toxicity; Survival and feeding rate.

Introduction

In natural environments, organisms are frequently exposed to mixtures of pollutants and it is relatively uncommon to find sites polluted with one toxicant only (Walker *et al*. 2001). Additionally, climatic changes can act as another source of stress and along with chemical compounds can lead to cumulative and/or synergistic impacts on aquatic and terrestrial organisms (Wrona *et al.* 2006).

In aquatic systems, many physiochemical factors, such as pH, hardness, temperature, dissolved oxygen and flow rates, affect the bioavailability and toxic properties of a compound toward aquatic species (Rand and Petrocelli 1985). In sediments and soils, organic matter, pH and the percentage of clay, silt and sand, are some of the factors of major importance for chemical toxicity (Chapman *et al*. 2003). Besides, natural conditions like temperature or dissolved oxygen can also influence not only chemical reactions but also the sensibility of organisms towards chemicals. In several occasions they can be considered natural stressors.

For several decades, ecotoxicological studies were carried out with single chemical exposures and in strict controlled conditions of temperature, photoperiod and moisture (in soil cases). Results were afterwards transposed to real scenarios of multiple stressors contamination which could lead to wrong assumptions.

Increasing the awareness on mixture contamination brought into discussion the development of new tools for assessing combined toxicity. Theoretical models based on the two concepts for prediction of mixtures toxicity (concentration addition and independent action) have been recently applied by several authors

(Altenburger *et al*. 2000; Backhaus *et al.* 2000; Barata *et al*. 2006; Cassee *et al.* 1998; Jonker *et al.* 2004; Jonker *et al*. 2005).

The concentration addition (CA) concept is applied to compounds having similar modes of action (MoA) and assumes that the relative toxicity of the mixture is the same as the relative toxicity of the individual compounds.

The concept of independent action (IA) assumes that the chemicals produce their effects through different mechanisms of action that have no interaction with each other (Olmstead and LeBlanc 2005). The mathematically formula is based on probability of responses and is expressed as:

$$Y = u_{\max} \prod_{i=1}^{n} q_i(c_i)$$

where Y denotes the biological response, c_i is the concentration of chemical *i* in the mixture, $q_i(c_i)$ the probability of non-response, u_{max} the control response for endpoints and ∏ the multiplication function (Jonker *et al.* 2005).

These conceptual models use the Toxic Unit (TU) approach in which individual toxicant concentration is divided by the concentration that produces a x % of effect on the organism when the toxicant is present singly (Norwood *et al*. 2003).

However in real scenarios compounds may interact with one another, modifying the magnitude and sometimes the nature of the toxic effect. Interaction might happen in the toxicokinetic phase (processes of uptake, distribution, metabolism and excretion) or in the toxicodynamic phase (effects of chemicals in the receptor, cellular target, or organ) (Cassee *et al*. 1998).

Some deviations from these two conceptual models have also been reported, showing different response patterns such as those producing a more severe (synergism), or less severe (antagonism) effect, or those dependent from dose level (different deviations at high and low doses) or dose ratio (deviations differ from mixture composition) may occur (Jonker *et al.* 2005).

In this study, binary mixtures of nickel chloride and chlorpyrifos and the combination of these two chemicals with low (LT) and high temperatures (HT) were chosen in order to predict their toxic effect behaviour.

Comparatively to other divalent metals, nickel (Ni) has not been well studied in terms of toxicity, mode of action, and bioavailability (Keithly *et al*. 2004). However, it is know to be a calcium channel blocker (Lee *et al*. 1999; Zamponi *et al.* 1996) leading to changes on intracellular concentrations of Ca(II) followed by signal gene expression changes associated with cell growth, differentiation and apoptosis (Valko *et al.* 2005). Nickel is also referred as oxidative stress inducer (Chen *et al*. 2003) by increasing levels of free radicals in cells (Bal and Kasprzak 2002), causing depletion of glutathione (GSH) (Rodriguez et al. 1996), inducing lipid peroxidation (Chen *et al.* 2002) and leading to DNA damage through the generation of reactive oxygen species (ROS) (Lynn *et al*. 1997).

Chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridil) phosphorothioate] (CPF) is a widely used, broad spectrum organophosphorothioate (OP) pesticide that displays activity against a broad range of insect pests. This compound may enter in surface waters by runoff, spraydrift or accidental spills (Cowgill *et al*. 1991) and exhibit moderate persistence and low solubility in water (van Wijngaarden *et al.* 1993). The toxicity of CPF results from initial metabolic activation to form chlorpyrifos oxon, with subsequent inactivation of acetylcholinesterase (AChE) at neural junctions (Giesy *et al*. 1999), resulting in the accumulation of the neurotransmitter acetylcholine at the post-synaptic receptor and overstimulation of the organism's peripheral nervous system (Printes and Callaghan 2004). Upon exposure to sublethal concentrations of OP insecticides, inhibition of brain and muscle AChE has been observed within 24hours, which indicates that bioactivation occurred rapidly after exposure (Giesy *et al.* 1999). This OP insecticide requires an initial metabolic transformation in target tissue to form chlorpyrifos *oxon*, which is intrinsically less stable and has greater toxic activity (NRA - National Registration Authority for Agricultural and Veterinary Chemicals 2000).

Temperature affects organisms directly through altered metabolism and oxygen levels and indirectly by actual interaction with toxic process (Viswanathan and Murti 1989). Photosynthesis, aerobic respiration, growth, reproduction, metabolism and the mobility of organisms are all affected by changes in water temperature. Indeed, the rates of biochemical reactions usually double when

temperature is increased by 10ºC within the given tolerance range of an organism (Rand and Petrocelli 1985). Aquatic organisms, in particular the ectotherms like daphnids, can only survive within a particular temperature range. If temperature goes too far above or below the tolerance range for a given, its ability to survive may be compromised.

The organism test used, *Daphnia magna* Straus, is know to be sensitive to OPs (Diamantino *et al*. 1998; Guilhermino *et al*. 1996), nickel (Kszos *et al*. 1992) and to low/high temperatures (Reichwaldt *et al.* 2005). It is an easy species to culture and has a high reproduction rate which is an important feature for mixtures assessments since a high number of organisms are needed. Another advantage on the use of *Daphnia* is their clonal method of reproduction which facilitates repeatable and reproducible results (Munzinger and Monicelli 1992).

The main goal of this study was to evaluate the effects of binary mixtures of nickel and chlorpyrifos and the combination of these chemicals with low/high temperatures to the cladoceran *Daphnia magna*, using lethal (survival) and sublethal (feeding) exposures. Feeding behaviour has a direct influence on *Daphnia* physiological performance in terms of growth, metabolism and reproduction (Nogueira 1996) and is usually affected by toxicant's exposure (Allen *et al.* 1995; Barata and Baird 2000), underlying causal mechanisms of population responses to toxic chemicals (Jak *et al*. 1996).

Materials and Methods

Test-chemicals and test-organisms

The chemical substances chosen for this study were: nickel chloride hexahydrate (CAS no. 7718-54-9; Merck) and chlorpyrifos (CAS no. 2921-88-2, 98% purity; Cheminova A/S).

Exposure medium contamination was controlled by Liquid Chromatography-Mass Spectrometry (LC-MS) for CPF analysis (Alliance 2695 equipped with autosampler, degasser, and heater column purchased by Water), and by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) for nickel analysis. Two

samples of the lowest and highest concentration were analysed for both chemical compounds.

All experiments were carried out with the cladoceran *Daphnia magna* Straus, clone K6 (originally from Antwerp, Belgium), successfully cultured in our laboratory for two years. Cultures were maintained in 1L glass beakers with 800ml of ASTM hard water (ASTM, 1980), renewed three times a week, and daily fed with *Pseudokirchneriella subcapitata* (Korshikov) Hindak at a concentration of 3×10^5 cells/ml and also with an organic extract (Baird *et al.*, 1989). About 25 daphnids per beaker were kept in 16:8 h light: dark cycle and temperature of 20±1ºC. Neonates from the fifth (or sixth) broods were used to re-start new cultures.

A reference test with potassium dichromate ($K_2Cr_2O_7$) was also performed every three months, to test daphnids sensitivity.

Single screening toxicity tests

Immobilisation tests: chemicals

Immobilisation tests were performed in accordance with the OECD 202 guideline (OECD 2004) using only third to fifth brood neonates aged less than 24-hrs.

Six chemical concentrations plus a negative control (ASTM only), with five replicates each were used for the experimental setup. When solvent addition was needed, a positive control with the highest solvent concentration was added to this setup. In each replicate, five neonates were exposed in a 50ml test solution and no food was provided during the 48-hrs of the test (20±1ºC and a 16:8h light:dark photoperiod). The number of daphnids immobilized after gentile stirring after 24-hrs and 48-hrs of exposure were recorded and the LC_{50} values calculated. Values for conductivity, dissolved oxygen, pH and temperature were also recorded for test validation.

Chemical nominal concentrations ranged from 6-10mg/L for nickel and 0.1-2.5µg/L for chlorpyrifos.

Immobilisation tests: natural stressor

A similar test design was used to obtain survival responses at high (HT) and low temperatures (LT). Daphnids were randomly exposed to four LT, ranging from 5.3 - 16.3ºC and four HT ranging from 24 – 39.5ºC, in five replicates with five organisms each. The control in these experiments was the 20ºC temperature. To achieve the different temperatures, tests were placed and run on climatic chambers (KBW 720, Binder, Germany) and waterbaths (Haake DC10, Thermo Electron Corp., Germany). Daily internal temperature records were done in order to verify possible variations.

Feeding inhibition and post-exposure tests

Organisms (<24h old) were separated from the main cultures to other culture beakers and maintained at the same conditions until 4 to 5 days old (equivalent to the fourth instars). Organisms within this life stage can complete a feeding inhibition bioassay (24-hrs exposure + 4-hrs post-exposure) within a single moult cycle (McWilliam and Baird 2002), thus avoiding moulting interference in daphnids feeding, following observations made by McWilliam and Baird (2002).

The setup of these experiments was made with 5 replicates per treatment with 5 individuals per replicate. An ASTM hard water control (plus a solvent control, if needed) was also used in these experiments. The group of five daphnids were randomly placed in 170ml beakers with 100 ml of test solution and fed for 24hrs with *P. subcapitata* at a concentration of 5×10^5 cells/ml.

To determine the initial concentration, a blank set of 3 replicates (with algae and no daphnids), was added for each test concentration. All the test beakers were placed in the dark, to produce uniform feeding rates (Haney 1985), during the 24-hrs exposure time. After this 24hrs period daphnids were transferred to clean ASTM medium with food (± 5 x 10^5 cells/ml *P. subcapitata)* for a 4-hrs period (post-exposure) also in the dark and the feeding rate determined. At the end of exposure and post-exposure periods, each replicate was vigorously shaken to re-suspend cells and its absorbance values were determined at 440nm by spectrophotometry (Jenway 6505 Spectrophotometer UV-VIS).

Individual feeding rates (cells/individual/hour) for 24-hrs exposure and 4-hrs post-exposure were determined according to the method given by Allen *et al.* (1995).

Nominal concentrations used for nickel ion ranged from 0.75 – 7.50µg/L and for chlorpyrifos from 0.050–0.250µg/L.

Mixture toxicity

In the mixture or combined stressors experiments the number of replicates was decreased to allow the use of more treatments in each test, in order to obtain a reliable coverage of the response surface. This increases both reliability and power of the analysis (Jonker *et al.* 2004, 2005), as the analysis is based on a regression model and variances are calculated between data and model values.

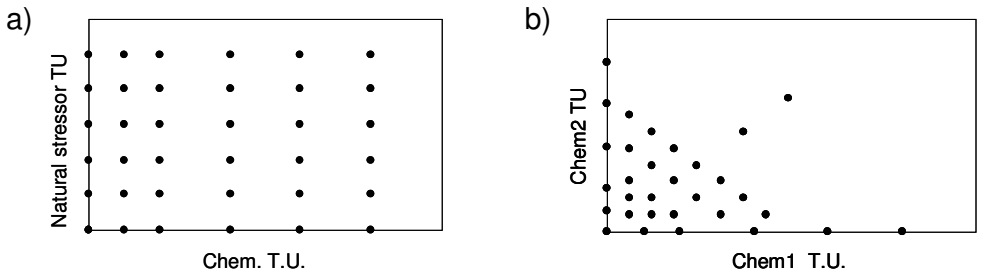

Fig.1 – A schematic design of toxic units combinations used for a) chemical vs. natural stressor combinations – factorial design and b) chemical vs. chemical mixtures – ray design.

Immobilisation tests: chemical mixtures

An experimental design, which includes simultaneously single evaluation of each compound and a set of twenty seven combinations, was chosen for binary mixtures testing. A full factorial design (Cassee *et al*. 1998) was used to assess the mixtures of nickel and chlorpyrifos (fig.1a).

Nominal concentrations used in single and combined evaluation ranged from 4 to 12mg/L and from 0.5 to 1.4µg/L for nickel and chlorpyrifos, respectively.

The 48-hrs LC_{50} values used for this calculation were obtained from previously made experiments with single exposures.

Immobilisation tests: combination of chemicals and natural stressor

Nominal concentrations of nickel and chlorpyrifos ranged from 4 to 12mg/L and from 0.5 to 1.4µg/L, respectively, were tested at six different temperatures (three HT and three LT). In order to obtain a dose-response curve for the statistical analysis, where the toxicity increases as the dose increases, a transformation of [20-x] was made for low temperatures, with 20 corresponding to the control temperature (20ºC) and x to the real low temperatures tested (0, 4, 8, 12 and 15ºC). For high temperatures, no transformation was needed since toxicity increases as the dose, i.e. the temperature, increases. Three and two replicates with five daphnids each were used for single and combined experiments, respectively. An experimental factorial design was followed (fig.1a).

Feeding inhibition tests

Similarly to acute experiments, the EC_{50} values obtained from single exposures were also applied to calculate mixtures compounds toxic units. Nominal concentrations of the mixtures were calculated based on expected toxic strengths of $\sum TU \leq 1$ with the exception of two equitoxic mixtures of $\sum TU=1.5$ and $\sum TU=2$ (corresponding to 0.75+0.75 and 1+1 TUs, respectively).

The concentrations of nickel used for the binary mixture with CPF ranged from 1 to 3mg/L for single and 0.3 to 2.4mg/L for combined evaluation. For CPF, the nominal concentrations used for this mixture ranged from 0.06 to 0.18µg/L and from 0.02 to 0.15µg/L for single and combined assessments, respectively.

At the low/high temperature exposures, the concentrations of nickel used for single and combined evaluation ranged from 0.5 to 4mg/L and for chlorpyrifos from 0.1 to 0.3µg/L. Again, real temperatures of 16, 18, 20, 22 and 24ºC were transformed by [24-x] or [20-x], where 24 and 20 corresponds to the maximum temperature of each exposure (HT and LT, respectively). One replicate with five daphnids plus a blank were used for each treatment. Both experimental designs showed in fig.1 were used in the feeding inhibition tests: a ray design for the chemical mixture, and a factorial design for the combination of different temperatures and chemical compounds.

Data analysis

The 48-hrs LC_{50} values for *D. magna* exposures were calculated by a probit analysis (Minitab, 2003) or in case of nonlinear data by a four parametric logistic curve (Systat, 2006).

In the feeding experiments, due to solvent (ethanol) use for chlorpyrifos exposures, statistically differences between controls were tested using t-test or a Mann-Whitney Rank test when normality failed. All significant differences were established at $p<0.05$ (Systat 2004).

To address the toxicity effects in the mixtures or combination experiments, the observed combined toxic effect was compared with an expected combined effect calculated from the single compound exposure toxicities, using the reference conceptual model, independent action (IA), described by Jonker *et al.* (2005). Deviations from IA model such as synergism/antagonism, dose-ratio and dose level were obtained by the addition of two parameters (a and b) to the equation (already described before) and tested within a nested framework and the best fit was chosen. The biological interpretation of these additional deviation parameters is listed in table 1 (adapted by Jonker *et al.* 2005). To find the best fit for the conceptual or deviation models, a Chi-square test based on residual sum of squares (SS) of deviations patterns was applied.

Table 1 – Interpretation of additional parameters (a and b) that define the functional form of deviation pattern from independent action (IA). Pos - positive; Neg - negative.

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

Results

Chemical analysis

In order to assess the contamination accuracy, nickel and chorpyrifos analysis were made. Real concentrations did not significantly differ from nominal concentrations. Thus, the results were based on nominal concentrations.

Single exposures

The 48-hrs LC_{50}/LT_{50} and 24-hrs EC_{50}/ET_{50} values resulted from acute and chronic (feeding) screening tests, respectively, are showed on table 2. As stated before, these values were used to calculate the TU values used to calculate mixture concentrations exposures, and also to control daphnids performance during mixture exposure.

In the chlorpyrifos bioassay, there was no difference between positive and negative controls in both acute and feeding inhibition tests ($p<0.05$).

Mortality occurrence in feeding tests was only observed at the two highest concentrations, during the 24hrs exposure period to CPF. Nickel and CPF showed a similar trend characterised by a decrease on feeding rates (Frates) as toxicant concentration increases (fig. 2a and b) on both exposure and post-exposure experiments.

Table 2 – 48-hrs LC_{50}/LT_{50}) and EC_{50}/ET_{50} values from acute and feeding inhibition screening tests on *D. magna*. Values in brackets refers to standard errors.

Chemical stressor		**48-hrs LC_{50} exposure**	**24-hrs EC_{50} exposure**	**4-hrs EC_{50} post-exposure**
Nickel (mg/L)		7.36 (0.23)	2.41 (0.30)	2.74 (0.15)
Chlorpyrifos (μg/L)		0.72 (3.70)	0.23 (0.27)	0.15 (0.01)
Natural stressor		**LT50-48hrs exposure**	**ET50-24hrs exposure**	**ET50-4hrs post-exposure**
Temperatures (ºC)	High	30.70 (228.77)	22.60 (0.24)	22.20 (4.16)
	Low	4.20 (1.08)	16.01 (0.0004)	16.10 (0.0003)

In the screening tests for different temperature exposures, an increase on feeding rates was observed as temperature increases, also for both periods (fig. 2c).

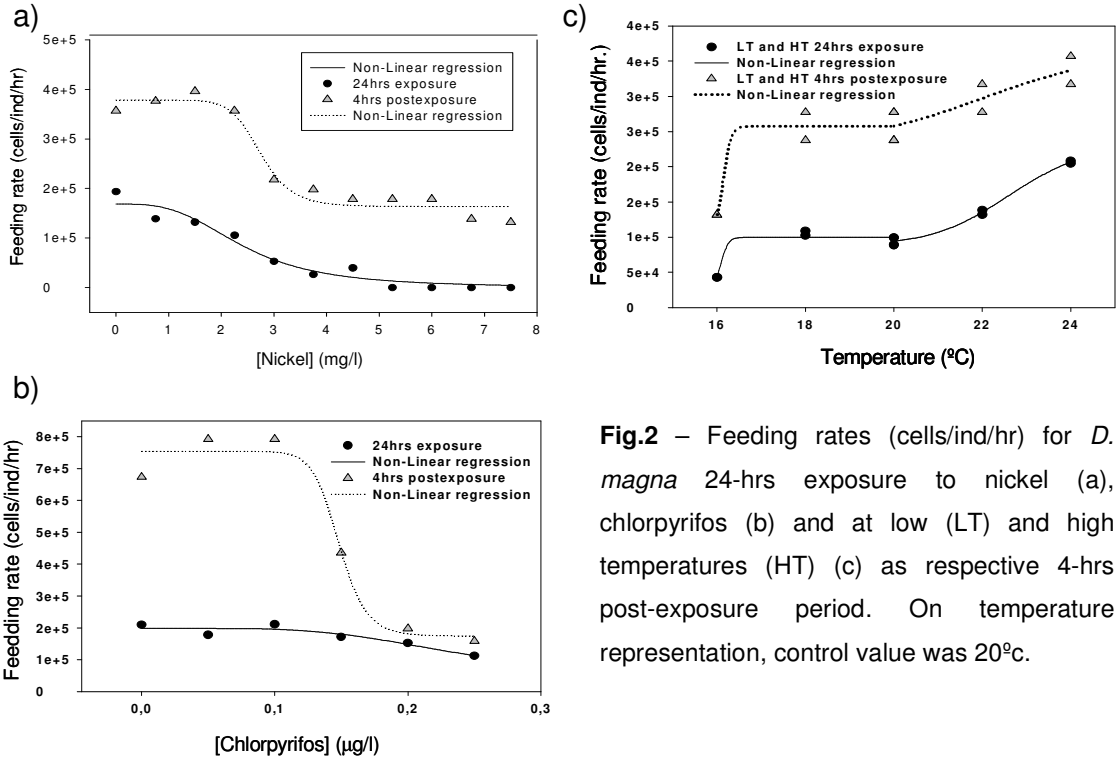

Fig.2 – Feeding rates (cells/ind/hr) for *D. magna* 24-hrs exposure to nickel (a), chlorpyrifos (b) and at low (LT) and high temperatures (HT) (c) as respective 4-hrs post-exposure period. On temperature representation, control value was 20ºc.

Mixtures exposures

Acute toxicity tests

In order to better understand the behaviour of nickel and CPF toxicity at low and high temperatures, LC_{50} and EC_{50} values of each chemical were calculated and presented in table 3 and 4, respectively.

Table 3 – 48-h LC_{50} values for nickel and chlorpyrifos (CPF) at low and high temperatures. Data obtained from acute tests on *D. magna*. Control temperature was 20ºC.

Temperature (ºC)	**0**	**4**	**8**	**12**	**15**	**20**	**25**	**31**	**35**
48-h LC_{50} for Nickel (mg/L)	0.00	51.30	30.60	8.15	4.46	7.21	7.21	4.46	0.00
48-h LC_{50} for CPF (µg/L)	0.00[a]	2.92	1.64	1.00	0.88	>1.40	0.50	0.06	0.00[b]

[a] - Value reported to a temperature of 2ºC. [b] - Value reported to a temperature of 33ºC.

Table 4 – 24-h EC_{50} and 4-h EC_{50} values for exposure and post-exposure, respectively, of nickel and chlorpyrifos (CPF) at low and high temperatures. Data obtained from feeding tests on *D. magna*. Control temperature was 20ºC.

Temperature (ºC)	**16**	**18**	**20**	**22**	**24**
24-h EC_{50} for Nickel (mg/L) exposure	2.72	1.75	3.62	1.67	1.76
4-h EC_{50} for Nickel (mg/L) post-exposure	1.36	2.04	2.42	2.58	3.96
24-h EC_{50} for CPF (µg/L) exposure	0.0051	0.14	0.36	2.65	0.09
4-h EC_{50} for CPF (µg/L) post-exposure	0.13	>>0.30	0.23	n.d	n.d

n.d – not determined

For the evaluation of response patterns of *D. magna* exposed to nickel and CPF mixtures, a significant and better fit was obtained with the deviation function for synergism (χ^2 test, P<0.05; SS= 26.98, *a*=-7.39, r^2=0.850) (fig.3a and b) when compared to IA conceptual model fit (SS= 60.20; r^2=0.666).

Data from combined experiments of high concentrations of nickel at high temperatures (HT) were fitted to IA model (SS=15.22; r^2=0.944) however changing the functions to assess deviations, a dose level dependency (χ^2 test, p<0.05; SS=3.63, *a*=-15.09, b_{DL} =1.06, r^2=0.986) was detected (fig. 4a and b, upper row).

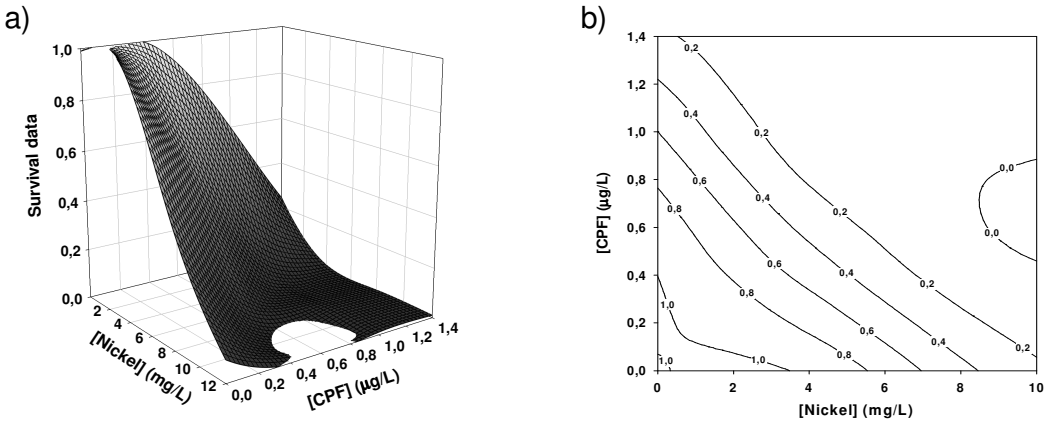

Fig.3 – Dose-response relationship for the binary mixture **CPF+Ni** showing a synergistic pattern for *D. magna*'s survival: a) 3D mesh and b) 2D isobolic surfaces. Control value for temperature is 20ºC.

The negative value for parameter *a* indicated synergism at low dose levels and antagonism at high dose levels while the value of parameter b_{DL} indicated that switching from synergism to antagonism occurred at the 94% effect level ($1/b_{DL}$ =1/1.06 = 0.94) (see table 1).

An identical behaviour was observed at low temperatures (LT) where a dose level-dependent deviation improve the data description significantly (χ^2 test, $p<0.05$; SS=96.59, a=-3.82, b_{DL} =5.59, r^2=0.691) (fig. 4a and b, lower row). Again, a more severe effect was observed at low dose levels while a decrease on toxicity was verified at high levels with switching occurring at doses where effect level is 19% ($1/5.59*LC_{50}$).

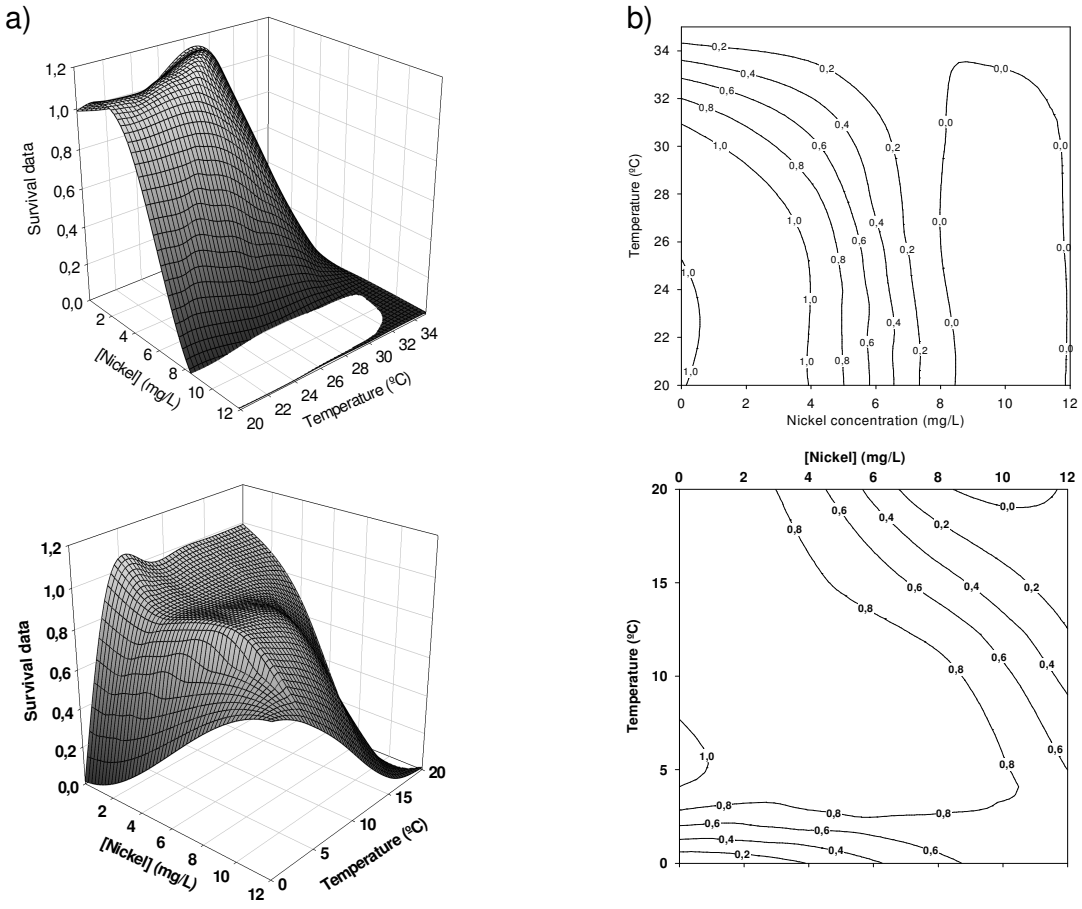

Fig.4– Dose-response relationship for the combination of **Ni + HT** (upper row) and **Ni + LT** (lower row) showing a dose level pattern for *D. magna*'s survival: a) 3D mesh and b) 2D isobolic surfaces. Control value for temperature is 20ºC.

In order to analyse data from the combinations of high doses/Levels of CPF and HT, IA model was tested and a more severe effect was resulted from the mixture, i.e. a significant synergism (χ^2 test, $p<0.05$; SS=7.05, *a*=-56.56, r^2=0.968) (fig. 5a and b, upper row).

When comparing data from CPF at LT exposures, also a synergistic deviation was verified (χ^2 test, $p<0.05$; SS=19.05, *a*=-31.29, r^2=0.911) (fig. 5a and b, lower row).

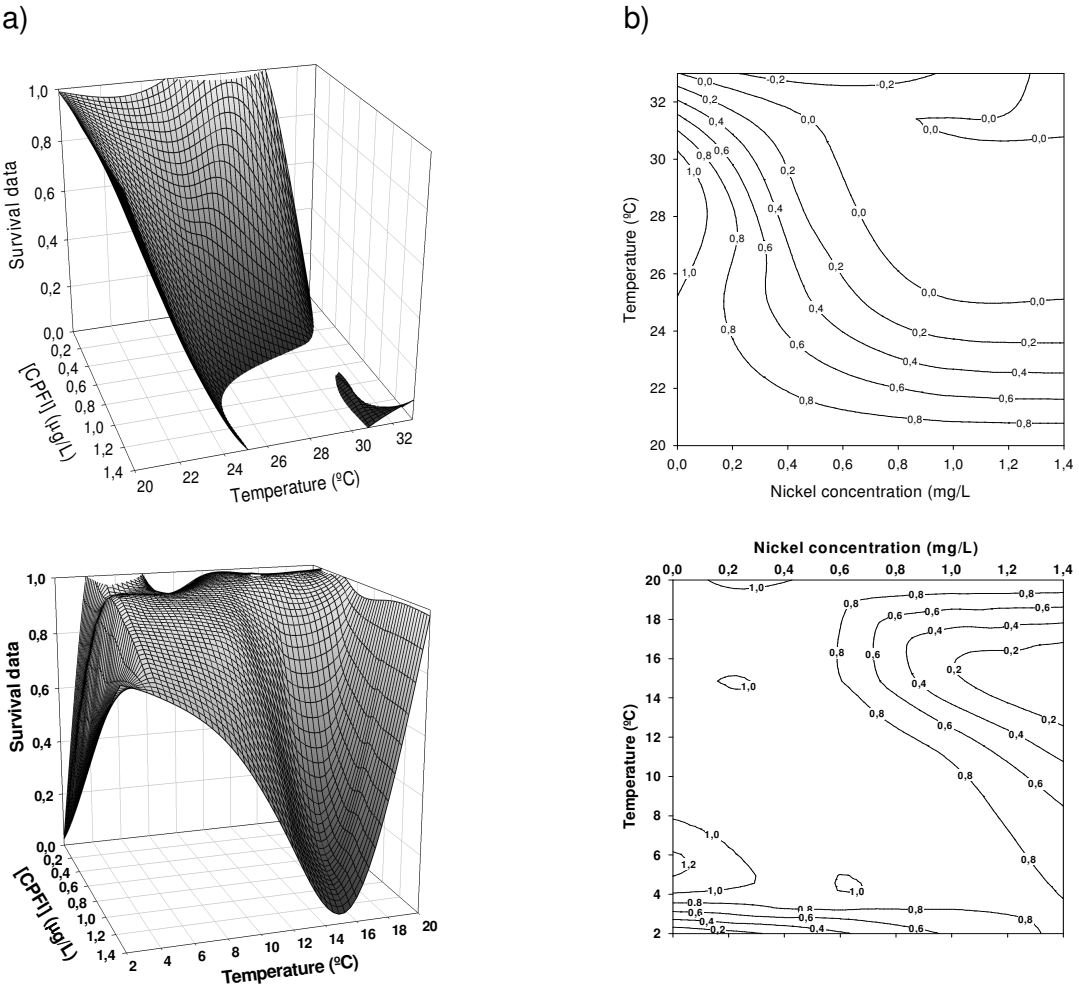

Fig.5 – Dose-response relationship for the combination of **CPF + HT** (upper row) and **CPF + LT** (lower row) showing a synergistic pattern for *D. magna*'s survival: a) 3D mesh and b) 2D isobolic surfaces. Control value for temperature is 20ºC.

Feeding inhibition tests

In the feeding inhibition tests, the same procedure analysis was used. Fitting the effect of nickel and chlorpyrifos mixture on daphnid's feeding activity after 24-hrs exposure to the IA model, an SS value of 1.09×10^{10} ($p<0.05$, $r^2=0.6493$) was obtained. However, while changing the function to antagonism/synergism (S/A), a little decrease on sum of squares was verified ($SS=9.1\times10^{9}$) with significant differences at $p<0.05$ (a=-1.95, $r^2=0.7065$), indicating synergism on feeding response (fig.6a and b).

a) b)

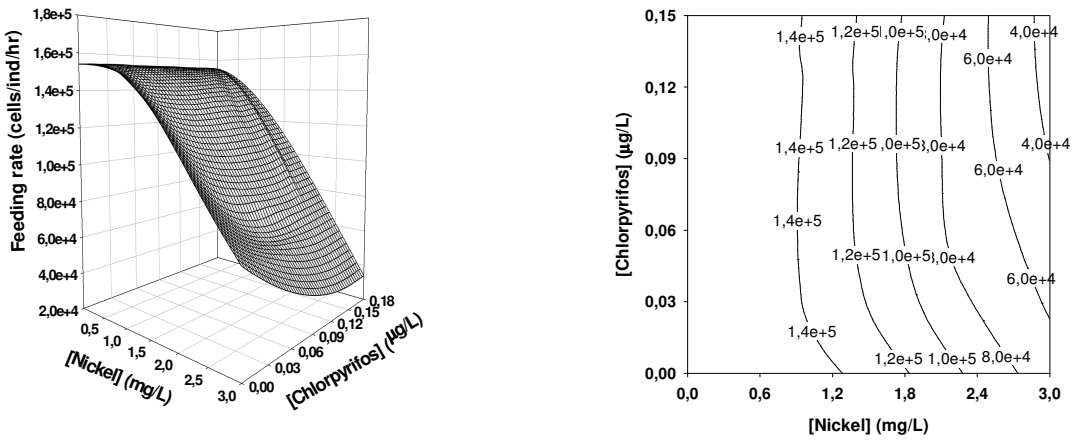

Fig.6 – Dose-response data from 24-hrs exposure period of *D. magna* to binary **Ni + CPF**, showing a synergistic pattern. Representations of feeding responses: a) 3D mesh and b) 2D isobolic surfaces.

In the post-exposure experiment, a dose ratio deviation was observed ($SS=1.15\times10^{11}$, a=-39005.77, b=39309.49, $r^2=0.808$) with a decrease on the mixture toxicity (antagonism) mostly when nickel was dominant on the mixture while a more severe effect was observed when the toxicity is mainly caused by CPF (fig.7a and b). The shift between antagonism and synergism occurred when [CPF] = 0.88 * [Ni].

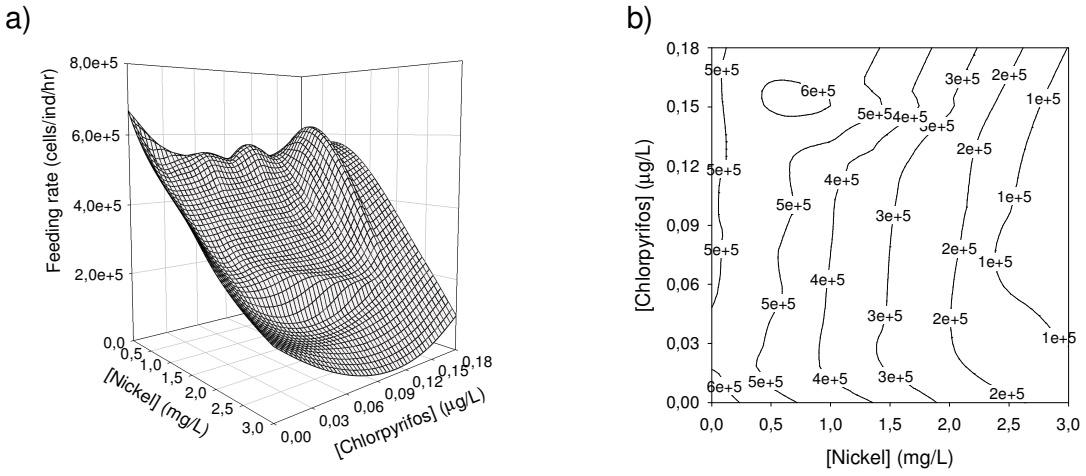

Fig.7 – Dose-response data from 4-hrs post-exposure period of *D.magna* to combined **CPF+Ni**, showing a dose ratio pattern. Representations of feeding responses: a) 3D mesh and b) 2D isobolic surfaces.

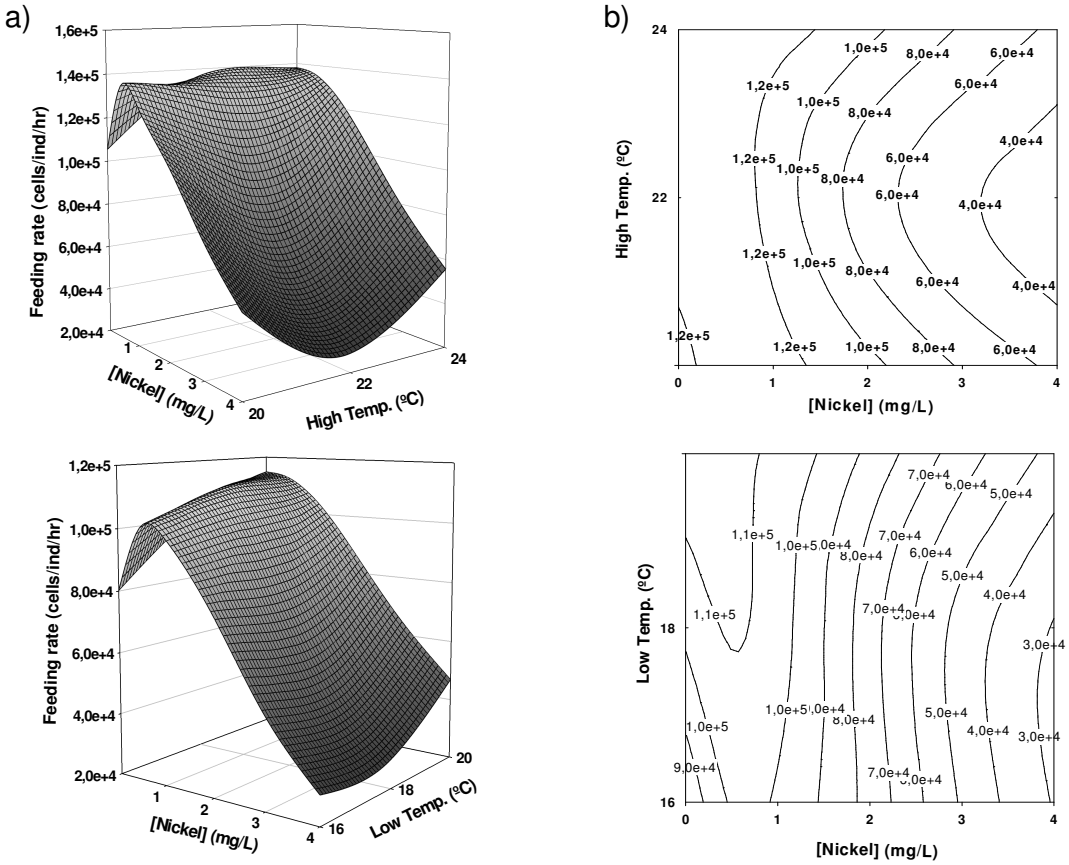

Fig.8 – Dose-response data from 24-hrs exposure of *D. magna* to the combinations **Ni+HT** (upper row) and **Ni+LT** (lower row) showing synergistic and a dose ratio patterns. Representations of feeding responses: a) 3D mesh and b) 2D isobolic surfaces. Control value for temperature is 20ºC.

Feeding data from the 24-hrs exposure period to the combination of nickel and HT was fitted to the independent action reference model (SS=5.67×10^9, r^2=0.786), however in order to assess S/A deviations occurrence, a decrease on sum of squares was verified (SS=3.89×10^9) with significant differences at $p<0.05$. Parameter *a* was negative, indicating synergism (*a*=-2.78, r^2=0.853) (fig.8a and b, upper row).

Post-exposure data were fitted to the IA model (SS=1.01×10^{11}, r^2= 0.707) but deviations were assessed resulting in a significant antagonism on feeding response (χ^2 test, $p<0.05$; SS=4.74×10^{10}, *a*=11024.27, r^2=0.863) as showed in fig. 9a) and 9b), upper row.

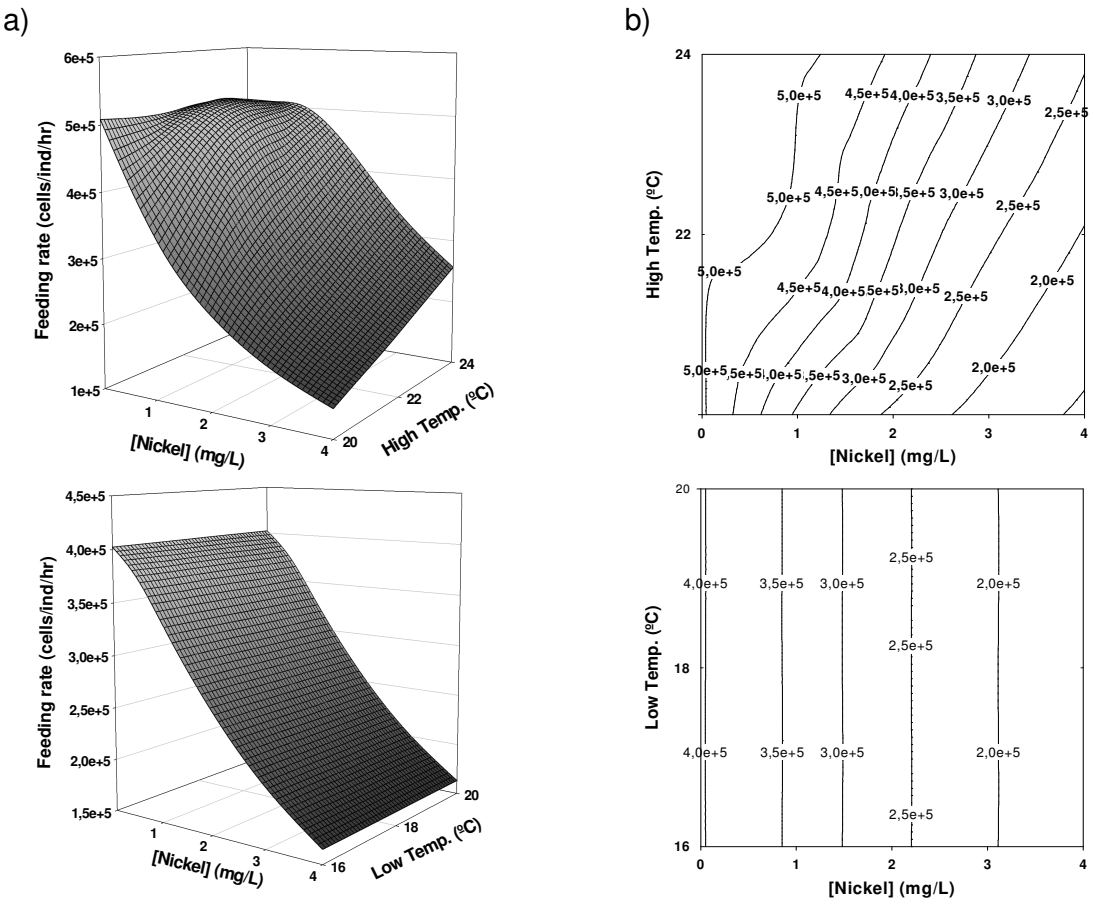

Fig.9 – Dose-response data from 4-hrs post-exposure period of *Daphnia magna* to combined **Ni+HT** (upper row) and **Ni+LT** (lower row), illustrating a synergistic deviation and no deviation from IA model, respectively. Representations of feeding responses: a) 3D mesh and b) 2D isobolic surfaces. Control value for temperature is 20ºC.

At low temperatures the same deviation from IA model were found with a SS value of 1.57×10^{9} and significant differences at $p<0.05$ (a=6.53, b=-10.66, r^2=0.901) (fig. 8a and b, lower row). Again, a more severe effect was observed when nickel was dominant as a stressor while a decrease on toxicity was mainly caused by LT (fig.8a and b, lower row).

In the post-exposure period it was observed that each stressor acted independently from the other when combined ($p<0.05$, SS=2.70×10^{10}, r^2=0.837) (fig. 9a and 9b, lower row).

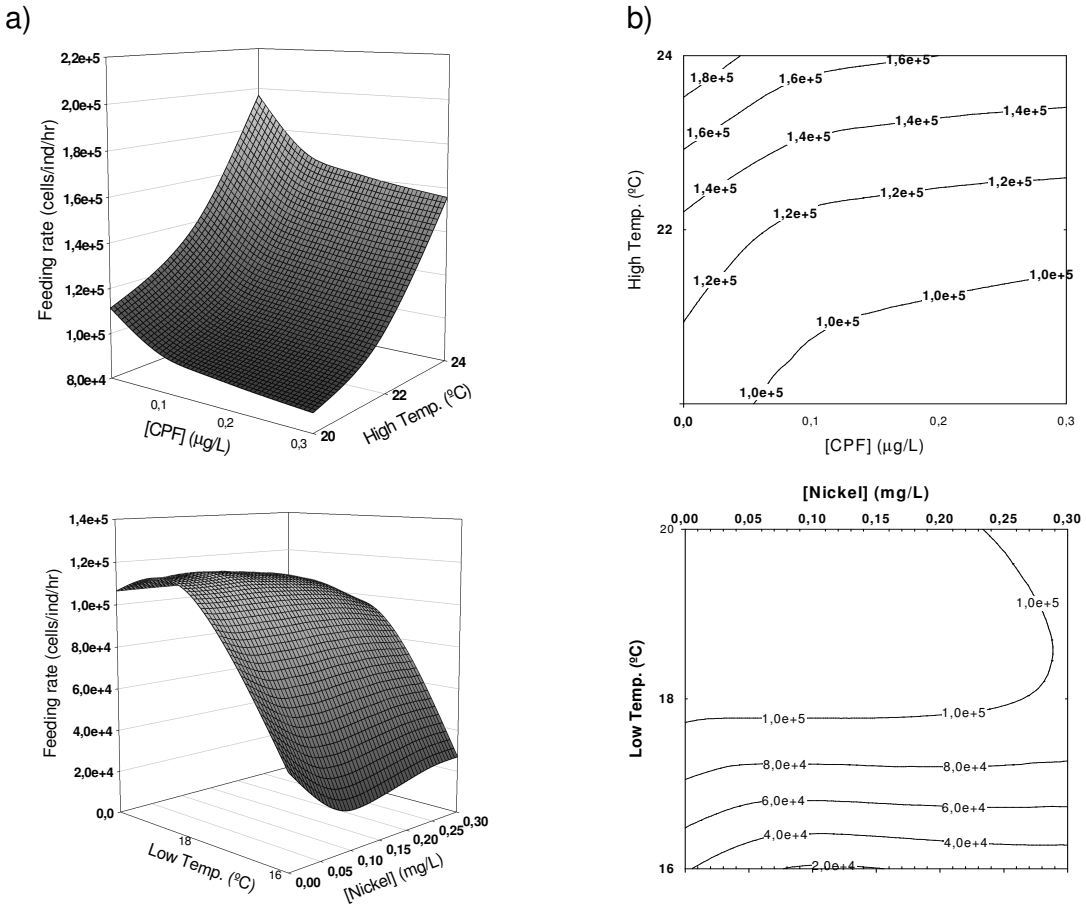

Fig.10 – Dose-response data from 24-hrs exposure period of *Daphnia magna* to combined **CPF+HT** (upper row) and **CPF+LT** (lower row), illustrating a no deviation and a dose ratio deviation from IA model, respectively. Representations of feeding responses: a) 3D mesh and b) 2D isobolic surfaces. Control value for temperature is 20ºC.

Data from 24-hrs feeding exposure of D. magna to chlorpyrifos and HT was fitted to the IA model (SS=2.75×109, r2=0.896) without significant deviations (fig.10a and b, upper row). Post-exposure data did not fit the IA model (SS= SS=3.51×1010, r2=0.097) neither the different deviations (p>0.05).

At low temperatures, feeding response on the exposure period to CPF showed to be dose ratio dependent (χ^2 test, P<0.05; SS=1.37×10^9, *a*=-7.45, *b*=15.56, r^2=0.951) with a less severe effect when chlorpyrifos was dominant in this combination (fig. 10a and b, lower row). After fitting the post-exposure data, no deviations were obtained from IA model (SS=3.99×10^{10}, r^2=0.757) indicating an independency on the recovery patterns of daphnids after the exposure of this combination (fig. 11a and b).

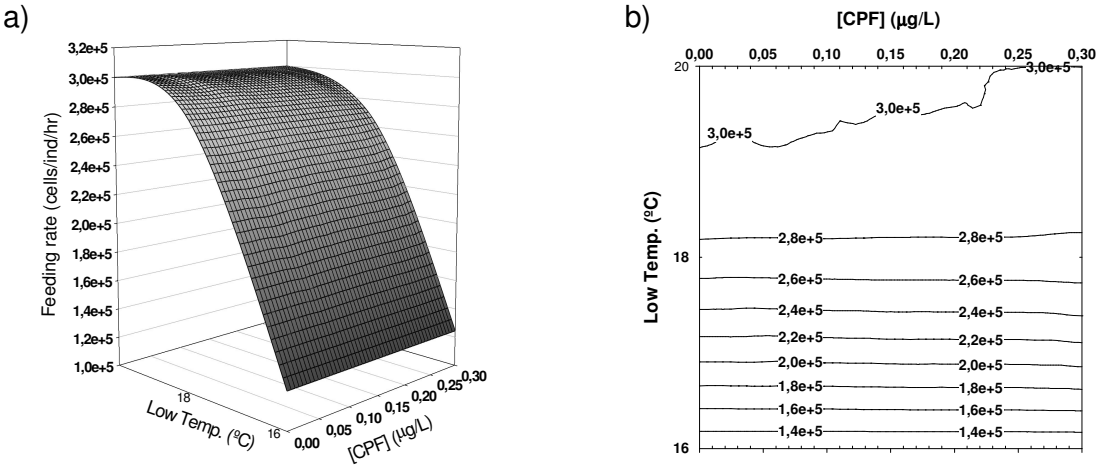

Fig.11 – Dose-response data from 4-hrs post-exposure period of *Daphnia magna* to combined **CPF+LT**, illustrating a no deviation from IA model. Representations of feeding responses: a) 3D mesh and b) 2D isobolic surfaces. Control value for temperature is 20ºC.

Discussion

Single stressor exposures

Although nickel is a widely used metal there is few data available on the toxicity to *D. magna*. Khangarot and Ray (1989) tested and ranked the immobilization of *Daphnia magna* caused by twenty-three metal ions and obtained

a 48-hrs LC_{50} value for nickel of 7.5mg Ni/L, very similar to the value calculated in our study (7.36mg Ni/L). A similar value of 6.48mg Ni/L for 48-hrs LC_{50} was reported by Wong (1992) when testing the effects of several metals on survival of the cladoceran *Moina macropoda*. In the study of Pane et al. (2003) on the toxicity mechanism of waterborne Ni to *Daphnia magna* obtained a value of 1.07mg Ni/L, but this study was carried out in soft water (60-70mg $CaCO_3$/L), while our daphnids were exposed to chemicals in ASTM hard water (140mg $CaCO_3$/L). This might explain the differences in the results because the increase of hardness has been reported as a factor that decreases nickel toxicity (Pane *et al*. 2004).

An expected reduction of the feeding rate was observed when daphnids were exposed to sublethal levels of nickel. In addition, it was also observed that daphnids could not recover in the post-exposure period showing a feeding inhibition patterns, also typical from metals action (Taylor *et al*. 1998).

The 48-hrs LC_{50} for chlorpyrifos (0.72µg CPF/L) obtained in our study was similar to that reported by Kersting and Van Wijngaarden (1992) when testing the effects of CPF on a microecosystem (1.0µg CPF/L). Barron and Woodburn (1995) for *Daphnia* sp. and Van Wijngaarden *et al*. (1993) for *Daphnia longispina* also reported similar 48-hrs LC_{50} values for CPF (0.1 – 0.5µg CPF/L and 1.0µg CPF/L, respectively).

In the sublethal assessment, not many studies have been conducted on the toxicity of CPF to cladoceran species. Some researchers state that CPF does not result in significant sublethal responses (Naddy *et al.* 2000). However, according to the Pesticide Action Network, toxic effects of CPF to zooplankton include accumulation, behaviour, and development problems, and effects on cells, enzymes, feeding, growth, and reproduction (Pesticide Action Network). In the present study, mortality occurred during feeding tests at the concentrations of 0.20 - 0.25µg/L as CPF. Since the 24-hrs LC_{50} was 1.93 µgCPF/L (data on 24-hrs acute toxicity not shown) and was obtain by testing the chemical without food we can conclude that the presence of algae increased chlorpyrifos toxicity. These findings are supported by Baladi (1998) in a study about the influence of food on the acute toxicity of chlorpyrifos to *D. magna* and by Naddy and Klaine (2001) when tested CPF toxicity to *Daphnia magna* in a pulse-exposure experiment. However, there is

no agreement between researchers on this issue. Rose *et al*. (2002) in their study with different food concentrations found that limiting food significantly would increase the toxicity of CPF to daphnids. Also Zalizniak and Nugegoda (2006) studied the effects of sublethal concentrations of CPF on successive generations of *D. carinata* and concluded that survival was higher during long-term exposure (with food) than in the acute toxicity testing (without food).

Temperature is an important factor that can impact the rate of most physiological processes mainly in ectotherms like daphnids. Differences in the environmental temperature may affect uptake, elimination and detoxification rates because it can induce changes in metabolic, locomotory, and feeding activity of organisms (Cairns *et al.* 1975; Donker *et al*. 1998; Fisher *et al*. 1999; Smit and VanGestel 1997). Heugens and co-workers (2003) assessed the effects of temperature on the cadmium toxicity to *Daphnia magna* and possible temperature-dependent toxicity processes, obtaining a 48-hrs LC_{50} for temperature of about 30-31ºC, which is similar to the 30.7ºC, obtained in our study. Kivivuori and Lahdes (1996) compared different heat tests in order to assess the effects of heat injury on *D. magna* and obtained a LC_{50} of 34.8ºC for 24-hrs of exposure. The difference between this value and that from our study is explained by the fact that longer exposure time generally decreased the LC_{50} value as reported by Heugens *et al*. (2003).

Mixture exposures

The analysis of adverse effects of chemical mixtures or combinations with natural stressors comprised two main conceptual models, concentration addition (CA) and independent action (IA), depending on the similarity of stressors' mode of action (MoA). The stressors used in this study (nickel, chlorpyrifos and temperature) exert different MoA, so the IA model and possible deviations were assessed.

We verified that in all acute exposures to mixtures of chemicals and combinations with low (LT) and high temperatures (HT), deviations from the IA conceptual model were found. In the sublethal exposures experiments only the combination of chlorpyrifos and high temperatures, fitted the IA model to explain

the joint toxicity behaviour. Also in the post-exposure experiments at low temperatures, the independent action model was the best to predict overall post-exposure effects (recovery).

Synergistic effects are usually associated to extreme environmental conditions, as HT, by directly and/or indirectly increase the toxicity of chemicals, as reported by several studies (e.g. Folt *et al*. 1999; Heugens *et al*. 2003; Heugens *et al*. 2006; Lewis and Horning 1991). A similar behaviour was observed in this study for acute exposures of nickel and chlorpyrifos at higher temperatures. Despite the significant dependent dose level deviation obtained after model fit (antagonism at high doses and synergism at low doses) for nickel assessment, and after calculating the 48-hrs LC_{50} at each HT (see table 3), a decrease on LC_{50} values as temperature rises was observed indicating a severe joint toxicity. The synergism obtained after model fit for CPF and HT combined exposure was also confirmed by the decrease of the 48-hrs LC_{50} values as temperature increases (Table 3). These overall effect is supported by Heugens et al. (2003), suggesting that an increase in toxicity may result from the combination of greater toxicant uptake and thermal stress caused by higher temperatures.

After fitting sublethal data from the combination of CPF and HT to the IA model it was observed that the two stressors act independently although an antagonistic and synergistic effect was observed at 20-22ºC and 22-24ºC, respectively, by the calculation of the EC_{50} values (see table 4). This is probably related to the fact that at 24ºC the feeding activity of *D. magna* reaches its maximum and consequently increasing the uptake of CPF (Koh *et al*. 1997). Another possibility is the increase on the production of haemoglobin typical at HT that leads to ROS formation and consequently to oxidative stress (Lamkemeyer *et al*. 2003; Seidl *et al*. 2005). Additionally Jager *et al.* (2007) showed that low doses of CPF might produce ROS, and that also might explain the synergism obtained.

On the acute assessment of CPF at LT, a significant synergistic deviation was observed. However, analysing the evolution of the 48-h LC_{50} at the different temperatures (see table 3), only at 15ºC it was verified a more severe effect. This means that at 15ºC, as the uptake rate was decreased, the overall effect was mainly caused by CPF. At lower temperatures than 15ºC, an antagonistic effect

was observed probably due to metabolism reduction and consequently by changes on toxicants uptake, elimination and detoxication rates (Tsui and Wang 2004). At the sublethal level, a synergistic effect was observed when the toxicity was caused by "high doses" (i.e. lower temperatures, higher stress) of LT and confirmed by the calculation of the EC_{50} (see table 4). This can be explained by the fact that a decrease in temperature leads to an increase in organism's sensitivity when referred to sublethal parameters (Smit and VanGestel 1997). A similar behaviour was verified for acute nickel and LT combined exposure after the calculation of the LC_{50} at the different temperatures (Table 3), and confirmed by the dose level dependency (antagonism at high doses and synergism at low doses) obtained after deviations assessment to the IA model.

For mixture toxicity assessment of nickel chloride and chlorpyrifos, synergistic effects at lethal and sublethal concentrations were found. Nickel mode of action is usually related to the production of reactive oxygen species (ROS) while chlorpyrifos is linked to acetylcholinesterase (AChE) impairments. However, in a recent study published by Jager *et al* (2007) it was suggested that chlorpyrifos MoA in low doses could be related to the formation of ROS and consequently to oxidative stress. Although not expected, the synergistic effect induced at low doses might be related to the production of reactive oxygen species induced by both compounds.

For post-exposure experiment an antagonistic effect was observed when CPF was dominant in the pre-exposure mixture composition. A possible reason for this fact can be the "regeneration" of AChE activity after chlorpyrifos has been removed from the test medium (Giesy *et al*. 1999).

Sublethal assessment of nickel chloride and HT showed an expected synergism probably as a result of the production of ROS by nickel toxicity enhanced by the increase on haemoglobin production typical from higher temperatures exposures (Lamkemeyer *et al*. 2003; Seidl *et al*. 2005). The antagonism obtained from nickel and HT post-exposure study, and confirmed by the calculation of the EC_{50} values (table 4), might be related to the temperature decrease experienced by the organisms from exposure to post-exposure period and the consequent re-stabilization of metabolic rates. The synergism verified for

feeding assessment of nickel at LT was unexpected since it was expected a decrease in the metabolic rate, including uptake, elimination and detoxification as well the locomotory and feeding activities of the organisms at LT, leading to a decrease in toxicity (Heugens *et al*. 2003). This result may be a statistical artifact since, at least for the nickel EC_{50} values (Table 4), there was a decrease (synergism) between 20ºC and 18ªC and after this, an increase (antagonism) between 18ºC and 16ºC.

Analysing the post-exposure data we concluded that the increase of temperature from exposure to post-exposure period as well the nickel toxicity caused on the exposure period to organisms, followed the independent joint action.

Interpretation of mixtures behaviours from post-exposure assessments must be done carefully once this nested model as well as the independent action and different deviations concepts are based on the direct toxicity caused by exposure to toxicants, while the post-exposure effects are reported to a pre-exposure period and consequently to the recovery of stress exposure.

Conclusions

The current study is an example of the variety of effects and behaviours that can result from stressors' combinations that are daily present in environment. Overall toxicity effects as synergism, antagonism, dose level and ratio dependent were observed and discussed.

Synergistic and dose level effects were obtained from acute exposures to stressors' combinations. Chlorpyrifos induced the more severe effects at the lethal level, since synergism was observed in all combinations with low/high temperatures.

During sublethal levels of exposure, nickel was the main inducer of high toxicity, as proved by the synergistic effects resulted from all the combinations that included this divalent metal.

Another important feature observed in this study was the synergism obtained on both lethal and sublethal assessments of nickel and chlorpyrifos mixtures. This result might be related to the recent findings obtained by Jager *et al.* (2007) in the springtail *Folsomia candida* about the production of ROS by low

doses of chlorpyrifos. Still, new studies on additive effects using the concentration addition model must be done before any conclusions.

Interpretation of mixtures behaviours from post-exposure assessments must be done carefully once this nested model as well as the independent action model and different deviations concepts, are based on the direct toxicity caused by exposure to toxicants, while the post-exposure effects are reported to a pre-exposure period. Still, this does not invalidate the use of this nested model to assess effects of a pre-exposure to combined stressors.

Synergistic effects associated at extreme environmental conditions, such as high temperatures, by directly and/or indirectly increasing the toxicity of chemicals were also observed in this study at both lethal and sublethal assessments, as already reported by several studies (Folt *et al.* 1999; Heugens *et al*. 2003; Heugens *et al*. 2006; Lewis and Horning 1991).

Nowadays the interpretation of combined effects is still difficult because chemicals and/or environmental stressors toxicity evaluation is not yet a routine in ecotoxicology resulting in a lack of data for comparasion.

Validation from toxicokinetics and toxicodynamics studies seems to be of crucial importance for this kind of approaches because it helps to understand some toxicological pathways involved in the exposure of complex mixtures. The detailed study of biomarkers such as AChE activity or those related with oxidative stress such as lipid peroxidation (LPO), catalase (CAT), glutathione S-transferase (GST) and total reduced glutathione (GSH) could also help to answer some of these questions.

Finally we can conclude that the nested model used here, and already described by Jonker *et al.* (2005), showed to be, in general, a good tool to evaluate the responses given by daphnids exposed to binary mixtures or stressors combinations.

This study intends to contribute to the new strategies that are being developed for the assessment of both human and ecological risks, based on the cumulative effects of combined exposures to multiple stressors (biological, chemical and physical).

Acknowledgments

The study was supported by the EU Integrated project NoMiracle (Novel Methods for Integrated Risk assessment of Cumulative Stressors in Europe; http://nomiracle.jrc.it) contract No. 003956 under the theme under the EU-theme "Global Change and Ecosystems" topic “Development of risk assessment methodologies”, coordinated by Dr. Hans Løkke at NERI, DK-8600 Silkeborg, Denmark.

References

Allen Y, Calow P, Baird DJ. 1995. A Mechanistic Model of Contaminant-Induced Feeding Inhibition in *Daphnia magna*. Environmental Toxicology and Chemistry 14(9):1625-1630.

Altenburger R, Backhaus T, Boedeker W, Faust M, Scholze M, Grimme LH. 2000. Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: Mixtures composed of similarly acting chemicals. Environmental Toxicology and Chemistry 19(9):2341-2347.

Backhaus T, Altenburger R, Boedeker W, Faust M, Scholze M, Grimme LH. 2000. Predictability of the toxicity of a multiple mixture of dissimilary acting chemicals to *Vibrio fischeri*. Environmental Toxicology and Chemistry 19(9):2348-2356.

Bal W, Kasprzak KS. 2002. Induction of oxidative DNA damage by carcinogenic metals. Toxicology Letters 127(1-3):55-62.

Barata C, Baird D, Nogueira A, Soares A, Riva M. 2006. Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. Aquatic Toxicology 78(1):1-14.

Barata C, Baird DJ. 2000. Determining the ecotoxicological mode of action of toxicants from measurements on individuals: results from short duration chronic tests with *Daphnia magna* Straus. Aquatic Toxicology 48(2-3):195-209.

Barron MG, Woodburn KB. 1995. Ecotoxicology of chlorpyrifos. Reviews of Environ. Contamination and Toxicology, Vol 144. New York: Springer-Verlag. p 1-93.

Cairns J, Heath AG, Parker BC. 1975. Effects of Temperature Upon Toxicity of Chemicals to Aquatic Organisms. Hydrobiologia 47(1):135-171.

Cassee FR, Groten JP, van Bladeren PJ, Feron VJ. 1998. Toxicological evaluation and risk assessment of chemical mixtures. Critical Reviews in Toxicology 28(1):73-101.

Chapman PM, Wang FY, Janssen CR, Goulet RR, Kamunde CN. 2003. Conducting ecological risk assessments of inorganic metals and metalloids: Current status. Human and Ecological Risk Assessment 9(4):641-697.

Chen CY, Su YJ, Wu PF, Shyu MM. 2002. Nickel-induced plasma lipid peroxidation and effect of antioxidants in human blood: Involvement hydroxyl radical formation and depletion of alpha-tocopherol. Journal of Toxicology and Environmental Health- Part A 65(12):843-852.

Chen CY, Wang YF, Lin YH, Yen SF. 2003. Nickel-induced oxidative stress and effect of antioxidants in human lymphocytes. Archives of Toxicology 77(3):123-130.

Cowgill UM, Gowland RT, Ramirez CA, Fernandez V. 1991. The History of a Chlorpyrifos Spill - Cartagena, Colombia. Environment International 17(1):61-71.

Diamantino TC, Ribeiro R, Goncalves F, Soares A. 1998. METIER (modular ecotoxicity tests incorporating ecological relevance) for difficult substances. 5. Chlorpyrifos toxicity to *Daphnia magna* in static, semi-static, and flow-through conditions. Bulletin of Environmental Contamination and Toxicology 61(4):433-439.

Donker MH, Abdel-Lateif HM, Khalil MA, Bayoumi BM, Van Straalen NM. 1998. Temperature, physiological time, and zinc toxicity in the isopod *Porcellio scaber*. Environmental Toxicology and Chemistry 17(8):1558-1563.

Fisher SW, Hwang H, Atanasoff M, Landrum PF. 1999. Lethal body residues for pentachlorophenol in zebra mussels (*Dreissena polymorpha*) under varying conditions of temperature and pH. Ecotoxicology and Environmental Safety 43(3):274-283.

Folt CL, Chen CY, Moore MV, Burnaford J. 1999. Synergism and antagonism among multiple stressors. Limnology and Oceanography 44(3):864-877.

Giesy JP, Solomon KR, Coates JR, Dixon KR, Giddings JM, Kenaga EE. 1999. Chlorpyrifos: Ecological Risk Assessment in North American Aquatic Environments. Reviews in Environ Contam. Toxicol. 160. p 1-129.

Guilhermino L, Lopes MC, Carvalho AP, Soares A. 1996. Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere 32(4):727-738.

Haney JF. 1985. Regulation of Cladoceran Filtering Rates in Nature by Body Size, Food Concentration, and Diel Feeding Patterns. Limnology and Oceanography 30(2):397-411.

Heugens EHW, Kraak MHS, Admiraal W, Hendriks AJ, Jager T, Creyghton R, Van Straalen NM. 2003. Temperature-dependent effects of cadmium on *Daphnia magna*: accumulation versus sensitivity. Environ Sci Technol 37(10):2145-51.

Heugens EHW, Tokkie LTB, Kraak MHS, Hendriks AJ, van Straalen NM, Admiraal W. 2006. Population growth of *Daphnia magna* under multiple stress conditions: Joint effects of temperature, food, and cadmium. Environmental Toxicology and Chemistry 25(5):1399-1407.

Jager T, Crommentuijn T, Van Gestel CAM, Kooijman SALM. 2007. Chronic exposure to chlorpyrifos reveals two modes of action in the springtail *Folsomia candida*. Environ. Pollut. 145:452-458.

Jak RG, Maas JL, Scholten MCT. 1996. Evaluation of laboratory derived toxic effect concentrations of a mixture of metals by testing fresh water plankton communities in enclosures. Water Research 30(5):1215-1227.

Jonker MJ, Piskiewicz AM, Castella NII, Kammenga JE. 2004. Toxicity of binary mixtures of cadmium-copper and carbendazim-copper to the nematode *Caenorhabditis elegans*. Environmental Toxicology and Chemistry 23(6):1529- 1537.

Jonker MJ, Svendsen C, Bedaux JJM, Bongers M, Kammenga JE. 2005. Significance testing of synergistic/antagonistic, dose-level dependent, or dose ratio-dependent effects in mixture dose response analysis. Environmental Toxicology and Chemistry 24(10):2701-2713.

Keithly J, Brooker JA, DeForest DK, Wu BK, Brix KV. 2004. Acute and chronic toxicity of nickel to a cladoceran (*Ceriodaphnia dubia*) and an amphipod (*Hyalella azteca*). Environmental Toxicology and Chemistry 23(3):691-696.

Kersting K, Vanwijngaarden R. 1992. Effects of Chlorpyrifos on a Microecosystem. Environmental Toxicology and Chemistry 11(3):365-372.

Khangarot BS, Ray PK. 1989. Investigation of Correlation between Physicochemical Properties of Metals and Their Toxicity to the Water Flea *Daphnia magna* Straus. Ecotoxicology and Environmental Safety 18(2):109-120.

Kivivuori LA, Lahdes EO. 1996. How to measure the thermal death of *Daphnia*? A comparison of different heat tests and effects of heat injury. Journal of Thermal Biology 21(5-6):305-311.

Koh HL, Hallam TG, Lee HL. 1997. Combined effects of environmental and chemical stressors on a model *Daphnia* population. Ecological Modelling 103(1):19-32.

Kszos LA, Stewart AJ, Taylor PA. 1992. An evaluation of nickel toxicity to *Ceriodaphnia dubia* and *Daphnia magna* in a contaminated stream and in laboratory tests. Environmental Toxicology and Chemistry; Vol/Issue: 11; DOE Project:Pages: 1001-1012.

Lamkemeyer T, Zeis B, Paul RJ. 2003. Temperature acclimation influences temperature- related behaviour as well as oxygen-transport physiology and biochemistry in the water flea *Daphnia magna*. Canadian Journal of Zoology-Revue Canadienne De Zoologie 81(2):237-249.

Lee JH, Gomora JC, Cribbs LL, Perez-Reyes E. 1999. Nickel block of three cloned T-type calcium channels: Low concentrations selectively block alpha 1H. Biophysical Journal 77(6):3034-3042.

Lewis PA, Horning WB. 1991. Differences in acute toxicity test-results of three reference toxicants on *Daphnia* at two temperatures. Environmental Toxicology and Chemistry 10(10):1351-1357.

Lynn S, Yew FH, Chen KS, Jan KY. 1997. Reactive oxygen species are involved in nickel inhibition of DNA repair. Environ. and Mol. Mutagenesis 29(2):208-216.

McWilliam RA, Baird DJ. 2002. Postexposure feeding depression: A new toxicity endpoint for use in laboratory studies with *Daphnia magna*. Environmental Toxicology and Chemistry 21(6):1198-1205.

Minitab 2003. Minitab Incorporation Statistical Software, version Release 14.1.

Munzinger A, Monicelli F. 1992. Heavy-Metal Cotolerance in a Chromium Tolerant Strain of *Daphnia magna*. Aquatic Toxicology 23(3-4):203-216.

Naddy RB, Johnson KA, Klaine SJ. 2000. Response of *Daphnia magna* to pulsed exposures of chlorpyrifos. Environ. Toxicol. and Chemistry 19(2):423-431.

Naddy RB, Klaine SJ. 2001. Effect of pulse frequency and interval on the toxicity of chlorpyrifos to *Daphnia magna*. Chemosphere 45(4-5):497-506.

Nogueira A. 1996. Moddeling the physiological performance of *Daphnia* (Crustacea:Cladocera) under stress. PhD thesis. Stirling University, Stirling, UK.

Norwood WP, Borgmann U, Dixon DG, Wallace A. 2003. Effects of metal mixtures on aquatic biota: A review of observations and methods. Human and Ecological Risk Assessment 9(4):795-811.

NRA - National Registration Authority for Agricultural and Veterinary Chemicals. 2000. The NRA review of Chlorpyrifos. NRA Review Series 1:1-87.

OECD. 2004. OECD Guidelines for the Testing of Chemicals. Guideline 202: *Daphnia* sp., Acute Immobilisation Test, adopted April 2004.

Olmstead AW, LeBlanc GA. 2005.Toxicity Assessment of Environmentally Relevant Pollutant Mixtures Using a Heuristic Model. Integrated Environmental Assessment and Management 1(2):114-122.

Pane EF, McGeer JC, Wood CM. 2004. Effects of chronic waterborne nickel exposure on two successive generations of *Daphnia magna*. Environmental Toxicology and Chemistry 23(4):1051-1056.

Pane EF, Smith C, McGeer JC, Wood CM. 2003. Mechanisms of acute and chronic waterborne nickel toxicity in the freshwater cladoceran, *Daphnia magna*. Environmental Science & Technology 37(19):4382-4389.

Pesticide Action Network. www.pesticideinfo.org.

Printes LB, Callaghan A. 2004. A comparative study on the relationship between acetylcholinesterase activity and acute toxicity in *Daphnia magna* exposed to anticholinesterase insecticides. Environmental Toxicology and Chemistry 23(5):1241-7.

Rand GM, Petrocelli SR. 1985. Fundamentals of aquatic toxicology: Methods and applications. United States: Hemisphere Publishing,New York, NY; FMC Corp., Princeton, NJ. Pages: 666 p.

Reichwaldt ES, Wolf ID, Stibor H. 2005. Effects of a fluctuating temperature regime experienced by *Daphnia* during diel vertical migration on *Daphnia* life history parameters. Hydrobiologia 543:199-205.

Rodriguez RE, Misra M, Diwan BA, Riggs CW, Kasprzak KS. 1996. Relative susceptibilities of C57BL/6, (C57BL/6xC3H/He) F-1, and C3H/He mice to acute toxicity and carcinogenicity of nickel subsulfide. Toxicology 107(2):131-140.

Rose RM, Warne MSJ, Lim RP. 2002. Food concentration affects the life history response of *Ceriodaphnia dubia* to chemicals with different mechanisms of action. Ecotoxicol Environ Saf 51(2):106-14.

Seidl M, Pirow R, Paul R. 2005. Acclimation of the microcrustacean *Daphnia magna* to warm temperatures is dependent on haemoglobin expression. Journal of Thermal Biology 30(7):532-544.

Smit CE, VanGestel CAM. 1997. Influence of temperature on the regulation and toxicity of zinc in *Folsomia candida* (Collembola). Ecotoxicology and Environmental Safety 37(3):213-222.

Systat 2004. Systat Software, Incorporation. SigmaStat for Windows version 3.10.

Systat 2006. Systat Software, Incorporation. SigmaPlot for Windows version 10.0

Taylor G, Baird DJ, Soares AMVM. 1998. Surface binding of contaminants by algae: consequences for lethal toxicity and feeding to Daphnia magna Straus. Environ. Toxicol. Chem. 17:412 - 419.

Tsui MTK, Wang WX. 2004. Temperature influences on the accumulation and elimination of mercury in a freshwater cladoceran, *Daphnia magna*. Aquatic Toxicology 70(3):245-256.

Valko M, Morris H, Cronin MTD. 2005. Metals, toxicity and oxidative stress. Current Medicinal Chemistry 12(10):1161-1208.

Van wijngaarden R, Leeuwangh P, Lucassen WGH, Romijn K, Ronday R, Vandervelde R, Willigenburg W. 1993. Acute Toxicity of Chlorpyrifos to Fish, a Newt, and Aquatic Invertebrates. Bulletin of Environ Contamination and Toxicology 51(5):716-723.

Viswanathan PN, Murti CRK. 1989. Effects of temperature and humidity on ecotoxicology of chemicals - In: Bourdeau P. (ed.), Haines J.A. (ed.), Klein W. (ed.), Krishna Murti C.R. (ed.) Ecotoxicology and climate. New York: J. Wiley. p: 139-154.

Walker C.H, Hopkin SP, Silby RM and Peakall D.B. 2001. Principles of Ecotoxicology, 2nd edition. Taylor & Francis, London, UK.

Wong CK. 1992. Effects of Chromium, Copper, Nickel, and Zinc on Survival and Feeding of the Cladoceran *Moina macrocopa*. Bulletin of Environmental Contamination and Toxicology 49(4):593-599.

Wrona FJ, Prowse TD, Reist JD, Hobbie JE, Levesque LMJ, Macdonald RW, Vincent WF. 2006. Effects of ultraviolet radiation and contaminant-related stressors on Arctic freshwater ecosystems. Ambio 35(7):388-401.

Zalizniak L, Nugegoda D. 2006. Effect of sublethal concentrations of chlorpyrifos on three successive generations of *Daphnia carinata*. Ecotoxicology and Environmental Safety 64(2):207-214.

Zamponi GW, Bourinet E, Snutch TP. 1996. Nickel block of a family of neuronal calcium channels: Subtype- and subunit-dependent action at multiple sites. Journal of Membrane Biology 151(1):77-90.

lll. Prediction of mixtures toxicity on survival and feeding parameters of *D. magna*: evaluation of binary combinations of cadmium, carbendazim and low dissolved oxygen

Abel L. G. Ferreira*, Susana Loureiro, Pedro Serra and Amadeu M. V. M. Soares
Department of Biology & CESAM, University of Aveiro
3810-193 Aveiro, Portugal
*email: aferreira@bio.ua.pt

Abstract – Environmental contamination is often characterised by a combination of stress factors of various sources (biological, physical and chemical). The predictability of their joint effects is an important stage in environmental risk assessment procedures. The two main conceptual models for mixtures evaluation based on the effect of individual compounds, concentration addition (CA) and independent action (IA) and deviations to synergism/antagonism, dose-ratio and dose-level dependency are being used. The present study analysed the single and combined effects of cadmium (metal), carbendazim (fungicide) and low dissolved oxygen levels (natural stressor) on life-cycle parameters (survival and feeding) of the water flea *Daphnia magna* Straus. The results of the single exposures revealed an increase of acute and sublethal toxicity as concentrations of cadmium and carbendazim increases. At low dissolved oxygen levels both survival and feeding parameters were significantly affected ($p<0.05$). In the acute mixture exposure of cadmium and carbendazim a dose ratio dependency was observed with a higher toxicity when cadmium was dominant whereas at high doses of carbendazim a lower effect on survival was observed. At sublethal exposures an antagonistic deviation from IA model was observed for this mixture. The IA model showed to be adequate for toxicity prediction on acute exposures combinations with low DO levels where a synergistic behaviour was observed. However at sublethal exposures IA and CA models failed by underestimation. Validation from

toxicokinetics and toxicodynamics modelling studies should be made in the future as a way to understand toxicological pathways involved in complex mixtures/combinations exposures.

Keywords – Independent action; Concentration addition; Synergism/antagonism; Dose-ratio dependence; Cadmium; Carbendazim; Dissolved oxygen; *Daphnia magna*; Mixture toxicity; Survival and feeding rate.

Introduction

General sources of anthropogenic chemical contaminants are from industrial, agricultural and urban uses. Multiple sources of contaminant inputs result in complex mixtures of toxicants in water, soil and sediments (Lipnick *et al.* 1996).

However, other types of stressors are increasing awareness due to actual climate changes phenomenon. Environmental variables, such as temperature and dissolved oxygen (DO) are two agents of stress that can be associated with chemicals' toxic responses and, in several occasions, they can be considered natural stressors. DO concentrations can vary spatially and temporally mainly due to changes in organisms' respiration, photosynthesis products, atmospheric losses and gains, changes in pressure and temperature (Dodds 2002).

Unfortunately, most of our knowledge and understanding of the effects of chemical contaminants in aquatic organisms is based upon the effects of single compounds tested in laboratory, instead of complex environmental mixtures. But this reality is changing and the awareness and interest of scientists and regulatory policies in the toxicology and potential risks of combined exposures is growing. As a consequence, it is now widely recognized that consideration of adverse effects caused by exposure to complex mixtures must be an integral part of environmental and human health risk assessment (Groten 2000).

Theoretical models based on the two non-interaction concepts for prediction of mixtures toxicity, concentration addition (CA) and independent action (IA), have been recently applied by several authors (Altenburger *et al*. 2000; Backhaus *et al*.

2000; Barata *et al*. 2006; Cassee *et al*. 1998; Jonker *et al.* 2004; Jonker *et al.* 2005). Both concepts allow us to calculate expected mixture toxicity on the basis of known individual toxicities of the mixture components.

The concept of CA assumes that individual toxicants with the same mode of action (MoA) act upon the same biological system (i.e. same molecular target) and contribute to a common response in proportion to their respective toxicities (Groten 2000). This conceptual model is defined as a summation of the relative toxicities of the individual components in mixture, and is mathematically expressed as:

$$\sum_{n}^{i=1} \frac{C_i}{ECx_i} = 1$$

where C_i is the concentration of the chemical *i* in the mixture and ECx_i is the effect concentration of the chemical *i* that produces the same effect (*x*%) as the whole mixture. For survival data simply exchange ECx with LCx (lethal concentration).

For the mathematical formulation of the CA model is usually used a dimensionless concept called "toxic unit (TU)" defined as the ratio of the actual concentration (c) of a substance to the concentration that is needed to cause a certain effect ECx (Backhaus *et al.* 2004):

$$\mathrm{TU} = \frac{c}{\mathrm{EC}_x}$$

The IA principle relates to independent modes of action of the mixtures components, i.e. individual compounds do not interfere with each other during exposure, uptake and toxic action (Olmstead and LeBlanc 2005). The mathematically formula is based on probability of responses and is expressed as:

$$Y = u_{\max} \prod_{i=1}^{n} q_i(c_i)$$

where Y denotes the biological response, c_i is the concentration of chemical *i* in the mixture, $q_i(c_i)$ the probability of non-response, u_{max} the control response for endpoints and $\prod$ the multiplication function.

In addition to these non-interactive concepts, interactions between mixture compounds and alterations on the magnitude of the toxic effect might also occur. The mechanism behind these interactions may be of physicochemical and/or biological nature and interactions can take place at the toxicokinetic phase (processes of uptake, distribution, metabolism and excretion) or at the toxicodynamic phase (effects of chemicals on the receptor, cellular target, or organ) (Cassee *et al.* 1998).

As real-world chemical mixtures may occur in a variety of doses, a more complex response patterns need to be addressed, such as those producing a more severe (synergism), or less severe (antagonism) effect, or those dependent from dose level (different deviations at high and low doses) or dose ratio (deviations differ from mixture composition) in order to assure a correct assessment of mixture effects (Jonker *et al.* 2005).

In this study, binary mixtures of cadmium chloride and carbendazim, and the combination of these two chemicals with low dissolved oxygen (DO) levels, as natural stressor, were chosen in order to predict their toxic behaviour.

Cadmium (Cd) is referenced by the European Community as a priority hazardous substance in the field of water policy (European Commission 2001) and has been classified as a #1 category human carcinogen by the International Agency for Research on Cancer of USA (1997). It is an abundant but non-essential heavy metal that can lead to the disruption of cellular homeostasis, such as oxidative stress (Pinto 2003), DNA damages (Badisa *et al.* 2007), membrane depolarization and acidification of the cytoplasm (Conner and Schimid 2003).

Carbendazim (CBZ) is a systemic broad spectrum benzimidazole carbamate fungicide that affects nucleus division and inhibits the activity of the enzyme acetylcholinesterase (Cuppen *et al.* 2000). Under natural environmental conditions, CBZ is very stable, and has been frequently detected in surface waters, with decomposition half-lives of two months under aerobic conditions, and 25 months in the absence of oxygen (Tomlin 1994). It has been included by the

European Commission on a priority list of chemicals that are believed to affect hormone function (European Commission 1999).

Dissolved oxygen depletion was the natural stressor chosen for this study because anthropogenic impacts have increased the frequency, duration, and intensity of hypoxia conditions in aquatic systems, resulting in changes in community composition and often loss of diversity (Connolly *et al*. 2004). Low levels of oxygen (hypoxia) also impair physiological processes in *Daphnia* species, especially in their circulatory system (Paul *et al*. 1997) and metabolic rate (Wiggins and Frappell 2000) and induce haemoglobin synthesis (Gorr *et al*. 2004). Synergistic effects associated to low D.O levels were reported by Hanazato and Dodson (1995) by exposure the cladoceran *Daphnia pulex* to the pesticide carbaryl.

The main goal of this study was to evaluate the effects of binary mixtures of Cd and CBZ and the combination of these chemicals with dissolved oxygen depletion to the cladoceran *Daphnia magna* Straus on acute (survival) and chronic (feeding) exposures. Feeding behaviour is usually affected under toxic exposure (Allen *et al*. 1995; Barata and Baird 2000) and this process has a direct influence on *Daphnia* physiological performance in terms of growth, metabolism and reproduction (Nogueira 1996) and is also one of the underlying causal mechanisms of population responses to the presence of chemicals in water (Jak *et al.* 1996).

Materials and Methods

Test-chemicals and test-organisms

Chemical compounds used in this study were cadmium chloride (technical grade, CAS No. 10108-64-2, Sigma-Aldrich, USA) and carbendazim (97% purity, CAS No. 10605-21-7, Aldrich Chemical Corp., USA).

Exposure medium contamination was controlled by chemical analysis by Liquid Chromatography-Mass Spectrometry (LC-MS) for carbendazim (Alliance 2695 equipped with autosampler, degasser, and heater column purchased by Water) and by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) for

cadmium. Two samples of the lowest and highest concentration and twenty-five samples of both single and combined exposures were analysed for carbendazim and cadmium, respectively.

All experiments were carried out with the cladoceran *Daphnia magna* Straus, clone K6 (originally from Antwerp, Belgium), successfully cultured in laboratory conditions for two years. Cultures were maintained in 1L glass beakers with 800ml of ASTM hard water (ASTM 1980), renewed three times a week, and daily fed with *Pseudokirchneriella subcapitata* (Korshikov) Hindak at a concentration of 3×10^5 cells/ml and also with an organic extract (Baird *et al*. 1989). About 25 daphnids per beaker were kept in 16:8h light: dark cycle and temperature of 20±1ºC. Neonates from the fifth (or sixth) broods were used to replace the old cultures. In some cases, a 20L aquarium with about 200 daphnids was used to support organism's requirement on large test designs.

A reference test with potassium dichromate ($K_2Cr_2O_7$) was also performed at each three months, to test daphnids sensitivity.

Single toxicity tests

Immobilisation tests: chemicals

Immobilisation tests were performed in accordance with the OECD 202 guideline (OECD 2004) using only the third to fifth brood neonates aged less than 24-hrs.

Six chemical concentrations plus a negative control (ASTM only), with five replicates each were used for the experimental setup. When solvent addition was needed a positive control with the highest solvent concentration was added to this setup. In each replicate, five neonates were exposed in a 50ml test solution and no food was provided during the 48-hrs of the test (20±1ºC and a 16:8h light: dark photoperiod). The number of daphnids immobilized after gentile stirring after 24-hrs and 48-hrs of exposure were recorded and the LC_{50} values calculated. Values for conductivity, dissolved oxygen, pH and temperature were also recorded for test validation.

Chemical nominal concentrations ranged from 25-250µg/L for cadmium and 80-200µg/L for carbendazim.

Immobilisation tests: natural stressor

A similar test design was used to obtain survival responses at different dissolved oxygen (DO) levels. Daphnids were randomly exposed to five concentrations of DO, ranging from 0.10 to 1.90mg DO/L, in five replicates with five organisms each. A control concentration of 9mg DO/L was also added to these tests. For this experimental setup, 100ml gastight glass Schott® bottles were used as test vessels to assure that no exchange of atmospheric gases could occur. Additionally, all the bottles were completely filled with test solution to prevent any presence of air bubbles which could change DO concentrations during exposure. All the tests procedures were done inside a Controlled Atmosphere Chamber (model 855-AC, PlasLabs, USA), saturated with nitrogen gas. To achieve the different dissolved oxygen concentrations, nitrogen gas was also injected on the test medium (ASTM) and the DO concentration measured using an oxygen probe (WTW 330i oxygen meter, Germany).

Feeding inhibition and post-exposure tests

Organisms (<24h old) were separated from the main cultures to other culture beakers and maintained at the same conditions until 4 to 5 days old (equivalent to the fourth instars). Organisms within this life stage can complete a feeding inhibition bioassay (24-hrs exposure + 4-hrs post-exposure) within a single moult cycle (McWilliam and Baird 2002), thus avoiding moulting interference in daphnids feeding, following observations made by McWilliam and Baird (2002).

The setup of these experiments was made with 5 replicates per treatment with 5 individuals per replicate. An ASTM hard water control (plus a solvent control, if needed) was also used in these experiments. The group of five daphnids were randomly placed in 170ml beakers with 100 ml of test solution and fed for 24-hrs with *P. subcapitata* at a concentration of 5×10^5 cells/ml.

For the DO testing, five concentrations ranging from 2.00-6.00 mg DO/L, plus a control (9.00 mg DO/L), were used and the methodology previously described was applied. To determine the initial concentration, a blank set of 3 replicates (with algae and no daphnids), was made for each test concentration. All the test beakers were placed in the dark, to produce uniform feeding rates (Haney 1985), during the 24-hrs exposure time. After this, all the daphnids from each replicate were transferred to 50ml "clean" (i.e, no toxicant and with 9mg DO/L) ASTM hard water with *P.subcapitata* at a concentration of 5×10^5 cells/ml and allowed to feed for 4-hrs, also in the dark (post-exposure period). At the end of exposure and post-exposure periods, each replicate was vigorously shaken to re-suspend any settled cells and its absorbance values were determined at 440nm by spectrophotometry (Jenway 6505 Spectrophotometer UV-VIS).

Individual feeding rates (cells/individual/hour) for 24-hrs exposure and 4-hrs post-exposure were determined according to the method given by Allen *et al.* (1995).

Nominal concentrations used for cadmium ion ranged from 5 to 50µg/L and for carbendazim from 10 to 100µg/L.

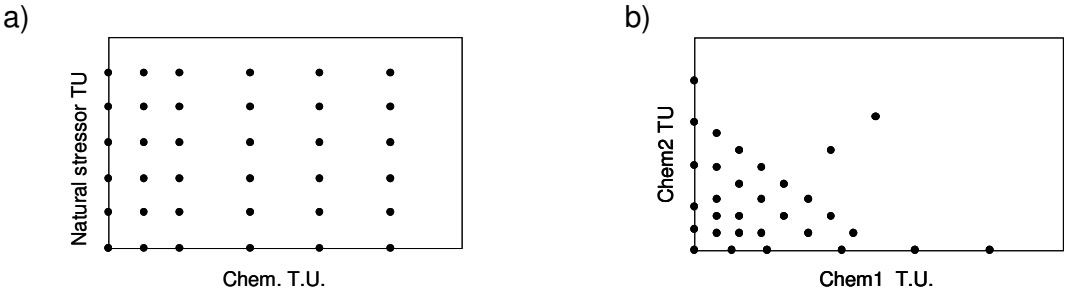

Fig.1 – A schematic design of toxic units combinations used for a) chemical *vs.* natural stressor combination – factorial design and b) chemical *vs.* chemical mixtures – ray design.

Mixture toxicity

In the mixture or combined stressors experiments the number of replicates was decreased to allow the use of more treatments in each test, in order to obtain a reliable coverage of the response surface. This increase both reliability and

power of the analysis (Jonker *et al.* 2004, 2005) as the analysis is based on a regression model and variances are calculated between data and model values.

Immobilisation tests: chemical mixtures

An experimental design, which includes simultaneously single evaluation of each compound and a set of twenty combinations, was chosen for binary mixtures testing. A ray design (Cassee *et al.* 1998) was used to assess the mixtures of Cd and CBZ (fig.1b). Nominal concentrations of the mixtures were calculated based on expected toxic strengths of 0.25 (0.125+0.125), 0.375 (0.125+0.25; 0.25+0.125), 0.5 (0.125+0.375; 0.25+0.25; 0.375+0.125), 0,75 (0.125+0.625; 0.25+0.5; 0.375+0.375; 0.5+0.25; 0.625+0.125), 1 (0.125+0.875; 0.25+0.75; 0.375+0.625; 0.5+0.5; 0.625+0.375; 0.75+0.25;0.875+0.125), 1.5 (0.75+0.75) and 2 (1+1) toxic units (TU). Nominal concentrations of Cd used in single evaluation ranged from 25 to 200µg/L and from 15 to 123µg/L in combined experiments and for CBZ from 25 to 200µg/L and 20 to 157µg/L in single and combined experiments, respectively. LC50 48-hrs values used for this calculation were obtained from previously made experiments with single stressors exposure. Four and two replicates with five organisms each were used for single and combined experiments, respectively.

Immobilisation tests: combination of chemicals and natural stressor

Five concentrations ranging from 25 to 250µg/L and 25 to 200µg/L of Cd and CBZ, respectively, were tested at six different dissolved oxygen concentrations. In order to obtain a dose-response curve for the statistical analysis, where the toxicity increases as the dose increases, a transformation of [9.0-x] was made, with 9.0 corresponding to the control concentration (9.0mg DO/L) and x to the real concentrations tested (0.5, 1.0, 1.5, 2.0 and 2.5 mg DO/L). Three and two replicates with five daphnids each were used for single and combined experiments, respectively. An experimental factorial design was followed (fig.1a).

Feeding inhibition tests

Similarly to acute experiments, the EC50 values obtained from single exposures were also applied to calculate mixtures compounds toxic units. Concentrations ranged from 10 to 50µg/L and 25 to 125 µg/L were used for binary mixtures and combinations of Cd and CBZ, respectively. Again, real DO concentrations of 2.0, 3.0, 4.0, 5.0 and 6.0 mg/L were transformed by [9.0-x], where 9.0mg DO/L corresponds to the control value. One replicate with five daphnids plus a blank were used for each treatment. Both experimental designs showed in fig. 1 were used in the feeding inhibition tests: a ray design for the chemical mixture, and a factorial design for the combination of DO and the chemical compounds.

Data analysis

The 48-hrs LC50 values for D. magna exposures were calculated by a probit analysis (Minitab, 2003) or in case of nonlinear data by a four parametric logistic curve (Systat, 2006).

In order to detect statistically differences between treated groups and control(s), feeding experiments data were analysed using a one-way analysis of variance (ANOVA) using the SigmaStat software (Systat, 2004). For data that followed a normal distribution, the post hoc multiple comparisons Dunnett's test was carried out when differences were obtained. When the normality test failed, a non-parametric Kruskal-Wallis test was used and multiple comparisons Dunn's method conducted. A solvent was used for carbendazim assessment, so differences between the negative and positive controls were tested using t-test or a Mann-Whitney Rank test when normality failed. All significant differences were established at $p<0.05$.

To address the toxicity effects of the mixtures, the observed combined toxic effect was compared with an expected combined effect calculated from the single compound exposure toxicities, using the reference conceptual models, concentration addition (CA) and independent action (IA) described by Jonker *et al.* (2005) according to their modes of action (MoA). Deviations from CA and IA models such as synergism/antagonism, dose-ratio and dose level were obtained

by the addition of two parameters (a and b) and tested within a nested framework and the best fit was chosen. The biological interpretation of these additional deviation parameters is listed in table 1 (adapted by Jonker *et al.* 2005). To find the best fit for the conceptual or deviation models, a Chi-square test based on residual sum of squares (SS) of deviations patterns was applied.

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). Pos - positive; Neg - negative. Adapted from Jonker *et al.* 2005.

Deviation Pattern	Parameter a (CA and IA)	Parameter b (CA)	Parameter b (IA)
synergism/antagonism (S/A)	**Pos:** antagonism		
	Neg: synergism		
Dose-ratio dependent (DR)	**Pos:** antagonism except for those mixture ratios where negative b value indicate synergism	**Pos b_i:** antagonism where the toxicity of the mixture is caused mainly by toxicant *i*	
	Neg: synergism except for those mixture ratios where positive b value indicate antagonism	**Neg b_i:** synergism where the toxicity of the mixture is caused mainly by toxicant *i*	
Dose-level dependent (DL)	**Pos:** 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
	Neg: 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

Chemical analysis

In order to assess the contamination accuracy, Cd and CBZ analysis were made. Real concentrations did not significantly differ from nominal concentrations. Thus, the results were based on nominal concentrations.

Table 2 – 48-h LC_{50} and EC_{50} values from 24hrs exposure and 4hrs post-exposure feeding inhibition tests with *D.magna* exposed to cadmium, carbendazim and dissolved oxygen. Values of standard errors in brackets.

Chemical stressor	**48-h LC_{50} exposure**	**24-h EC_{50} exposure**	**4-h EC_{50} post-exposure**
Cadmium (µg/L)	79.05 (6.47)	35.54 (0.02)	24.34 * (0.01)
Carbendazim (µg/L)	156.66 (3.70)	97.54 (0.15)	n.d
Natural stressor	**48-h LC_{50} exposure**	**24-h EC_{50} exposure**	**4-h EC_{50} post-exposure**
Dissolved oxygen (µg/L)	513.43 (1,934.06)	2,210.00 (0.01)	<< 2,000.00

n.d – not determined.
* - refers to concentrations on the exposure period.

Single exposures

The 48-hrs LC_{50} and 24-hrs EC_{50} values from single exposures in the immobilisation and feeding inhibition tests, respectively, are showed in table 2. As stated before, these values were used to calculate the TU values used to calculate the mixture concentrations exposures.

In the CBZ bioassay, there were no differences between positive and negative controls in both acute and feeding inhibition tests ($p<0.05$).

No mortality occurred in all feeding inhibition tests. On the 24-hrs period exposure for the feeding inhibition tests, Cd and CBZ showed a similar trend characterised by a decrease in daphnid's feeding rates (Frates) as toxicant concentration increased (fig. 2a and 2b). Statistically significant differences were found for both chemicals in the higher concentrations' exposures and LOEC values of 40 and 60µg/L were obtained for Cd and CBZ, respectively (fig. 2a and b). Post-exposure experiments showed an increase in the feeding activity for Cd with significant differences at 40, 45 and 50µg/L ($p<0.05$). A similar behaviour was observed for CBZ post-exposure with statistically differences at 90µgCBZ/L ($p<0.05$).

In the highest concentration of Cd post-exposure, feeding rate value was about 2.7-fold higher than the control and 36-fold higher than the value obtained during the exposure period at the same concentration. Comparing both exposure and post-exposure feeding rate values for the control treatments it was observed an increase of about 1.3-fold and significant differences were found ($p<0.05$).

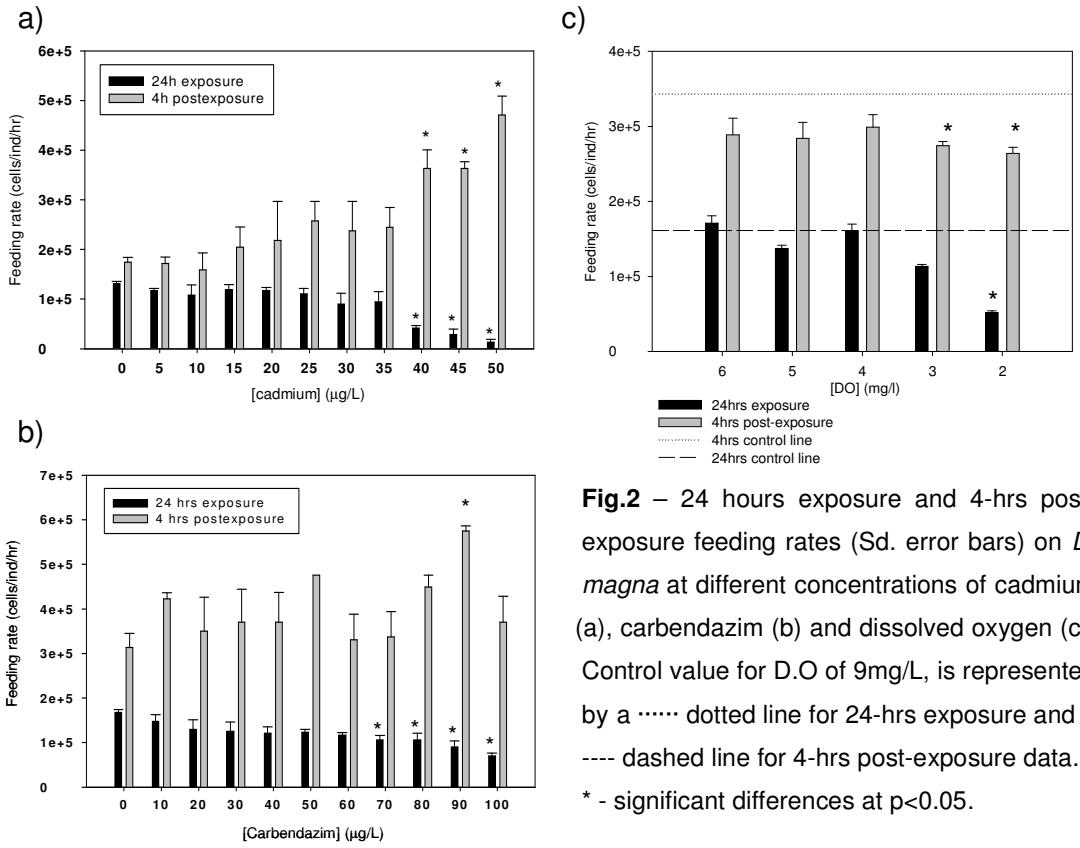

Fig.2 – 24 hours exposure and 4-hrs post-exposure feeding rates (Sd. error bars) on *D. magna* at different concentrations of cadmium (a), carbendazim (b) and dissolved oxygen (c). Control value for D.O of 9mg/L, is represented by a ····· dotted line for 24-hrs exposure and a ---- dashed line for 4-hrs post-exposure data. * - significant differences at $p<0.05$.

For CBZ, the feeding rate value for daphnids pre-exposed to 90µg CBZ/L was1.8-fold higher than the value found for the control, however when comparing this value with that obtained at the same concentration in the exposure period, no differences were found ($p<0.05$). Considering the control treatments, significant differences between exposure and post-exposure periods were found and an increase of about 3.2-fold was observed in the post-exposure period (fig.2b).

For the 24-hrs DO levels exposure a decrease in the feeding activity was also observed and a LOEC value of 2mg/L determined ($p<0.05$). After the 4-hrs

post-exposure period this tendency was maintained but with an increase on the LOEC value to 3mg/L (fig. 2c). An EC50 value was not possible to calculate but a 33% reduction in the feeding activity was observed at 2mg/L DO. Both control and the lowest DO concentration showed a significant increase on the feeding rates of about 2 and 5-fold, respectively, when daphnids were moved from the exposure to the post-exposure period ($p<0.05$).

Mixtures exposures

Acute toxicity tests

In order to better understand the behaviour of the Cd and CBZ toxicity at low D.O levels, LC50 and EC50 values of each chemical were calculated and presented in table 3 and 4, respectively.

Table 3 – 48-hrs LC_{50} values for cadmium and carbendazim at low dissolved oxygen levels (D.O). Data obtained from acute tests on *D. magna*. Control value for D.O was 9mg/L.

Dissolved oxygen (mg/L)	**9.0**	**2.5**	**2**	**1.5**	**1.0**	**0.5**
48-hrs LC_{50} for Cadmium (µg/L)	104.9	97.8	98.0	90.1	66.8	8.2
48-hrs LC_{50} for Carbendazim (µg/L)	145.3	54.1	73.1	103.0	68.7	28.2

Table 4 – 24-hrs EC_{50} and 4-hrs EC_{50} values for exposure and post-exposure, respectively, of cadmium and carbendazim, at low dissolved oxygen levels (D.O). Data obtained from feeding tests on *D. magna*. Control value for D.O was 9mg/L.

Dissolved oxygen (mg/L)	**9**	**6**	**5**	**4**	**3**	**2**
24-hrs EC_{50} for cadmium (µg/L) exposure	86.2	14.8	9.9	5.6	8.4	3.5
24-hrs EC_{50} for carbendazim (µg/L) exposure	136.9	n.d	28.6	22.9	24.4	3.5
4-hrs EC_{50} for Cadmium (µg/L) post-exposure	51.2	43.6	20.2	30.2	11.2	11.0
4-hrs EC_{50} for Carbendazim (µg/L) post-exposure	53.9	14.07	n.d	n.d	n.d	0.1

n.d –not determined

To understand daphnids' behaviour to mixture exposure, the reference models of combined toxicity CA and IA were used depending on the similarity or dissimilarity (respectively) of targets and/or processes involved in when compounds act individually.

Data from the acute exposure of CBZ and Cd to daphnids were fitted with the conceptual model IA (SS=86.800; r^2=0.755). A significant and better fit was obtained afterwards with the deviation function for dose ratio dependency (χ^2 test, p<0.05; SS= 41.737, a=-2.67, b= -13.93, r^2=0.882). In this case an increase on the mixture toxicity (synergism) was observed and explained mostly when cadmium was the dominant chemical in the mixture (fig.3a and b).

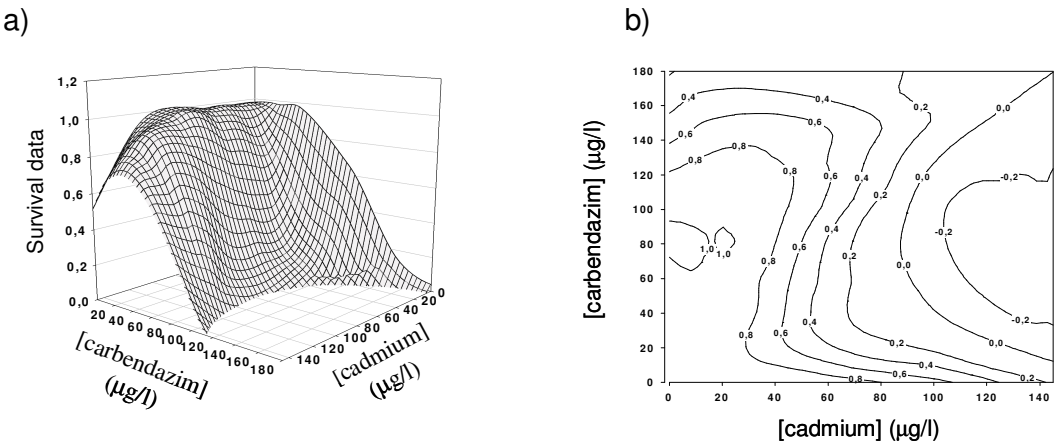

Fig.3 – Dose-response relationship for the binary mixture **Cd+CBZ** showing a dose ratio deviation from the IA model for *D. magna*'s survival: a) 3D mesh and b) 2D isobolic surfaces.

Data from combined experiments involving high concentrations of Cd and extremely low concentrations of DO were applied for both reference mixtures models since both stressors can cause oxidative stress on *Daphnia* cells in different ways. A significant antagonistic effect (χ^2 test, P<0.05; SS=48.65, a=1.60, r^2=0.893) was detected when compared to the CA model fitting (SS=166.55; r^2=0.635) (fig. 4a and b). An opposite behaviour was observed for IA reference model (SS=42.69; r^2=0.907) where a synergistic effect (χ^2 test, p<0.05; SS=38.27, a=-1.96, r^2=0.916) (fig. 4a and b) showed to be the best fit to our data.

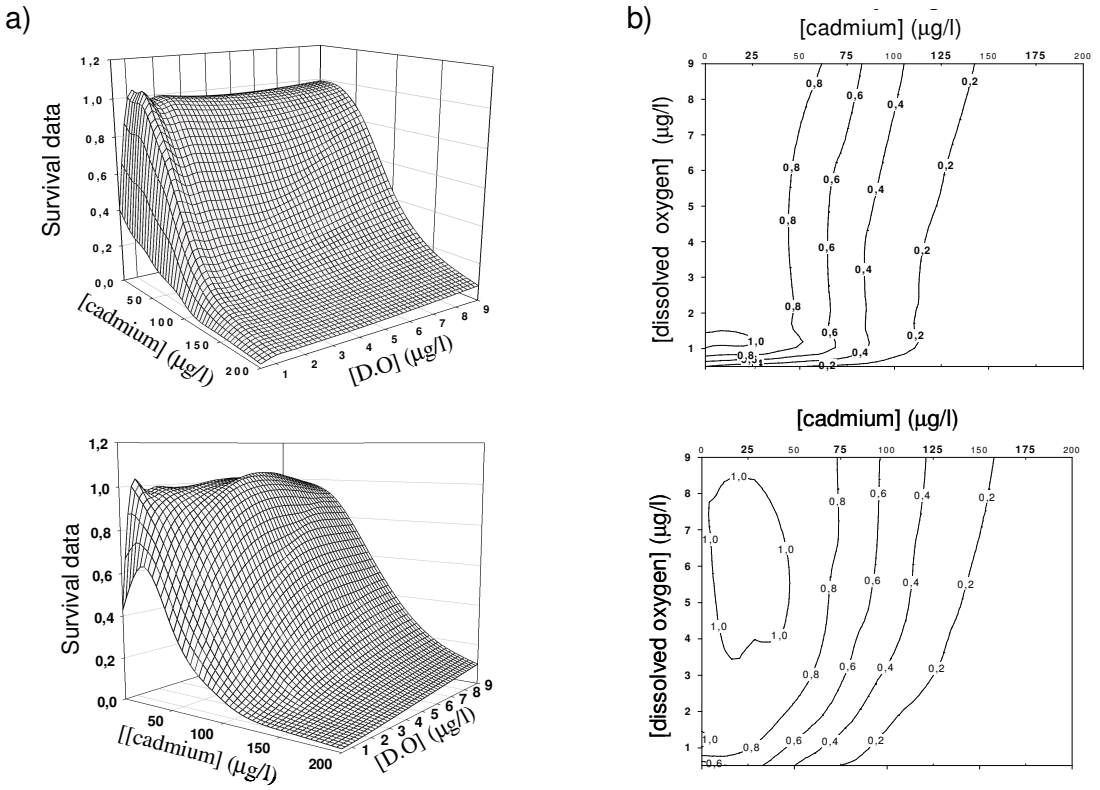

Fig.4 – Dose-response relationship for the combination of **Cd+D.O**. showing a synergistic deviation from the IA model (upper row) and an antagonistic deviation from the CA model (lower row) for *D. magna*'s survival: a) 3D mesh and b) 2D isobolic surfaces. Control value for D.O is 9mg/L.

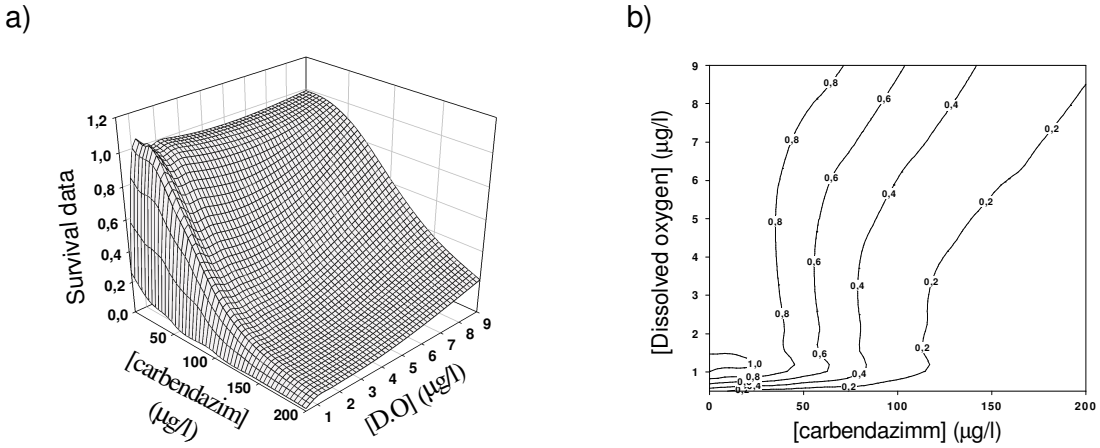

Fig.5 – Dose-response relationship for the combination of **CBZ+D.O**. showing a synergistic deviation from the IA for *D. magna*'s survival: a) 3D mesh and b) 2D isobolic surfaces. Control value for D.O is 9mg/L.

In order to analyse data resulted from the combination of high concentrations of CBZ and extremely low DO levels, IA model was tested but a more severe effect was observed, resulting in a significant synergism (χ^2 test, $p<0.05$; SS=108.12, a=-3.83, r^2=0.792) (fig. 5a and b).

Feeding inhibition tests

In the feeding inhibition experiments, the same procedure analysis was used. Comparing the effect data of CBZ and Cd mixture on feeding activities, after 24-hrs exposure, to the IA model predicted data, an SS value of 1.67×10^{10} (r^2=0.664) was obtained. However, while changing the function to assess antagonism/synergism (S/A) deviation, a little decrease on sum of squares was verified (SS=8.27×10^{9}) with significant differences at $p<0.05$ (a=4.35, r^2=0.833), indicating antagonism on feeding response (fig.6a and b). In the post-exposure experiment, IA model fit our data but not very accurately (SS=1.73×10^{11}, r^2=0.410).

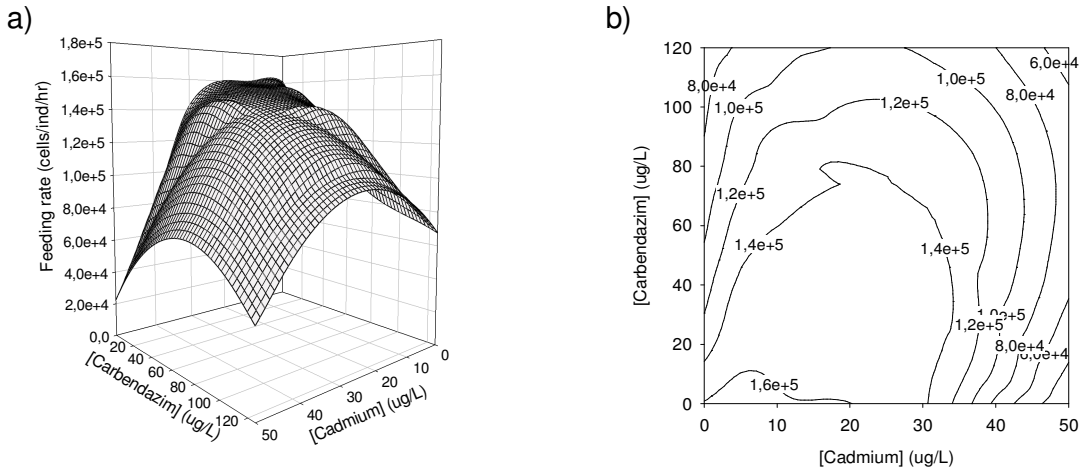

Fig.6 – Dose-response data from 24-hrs exposure for the binary mixture **CBZ+Cd**, showing an antagonistic deviation from the IA model for *Daphnia magna*'s feeding response: a) 3D mesh and b) 2D isobolic surfaces.

Feeding data from the 24-hrs exposure period to the combination of Cd and DO showed similar fitting to both CA (SS=2.85×10^{10}, r^2=0.783) and IA (SS=2.91×10^{10}, r^2=0.779) reference models. Again, in order to assess S/A deviations occurrence

from the CA model, a decrease in the sum of squares was verified (SS=2.27×10^9) with significant differences at $p<0.05$ (a=0.53, r^2=0.827), indicating antagonism (fig.7a and b, upper row). The same antagonistic deviation was verified for IA model with a sum of squares value of 2.35×10^9 and significant differences at $p<0.05$ (a=1.34, r^2=0.821) (fig. 7a and b, lower row).

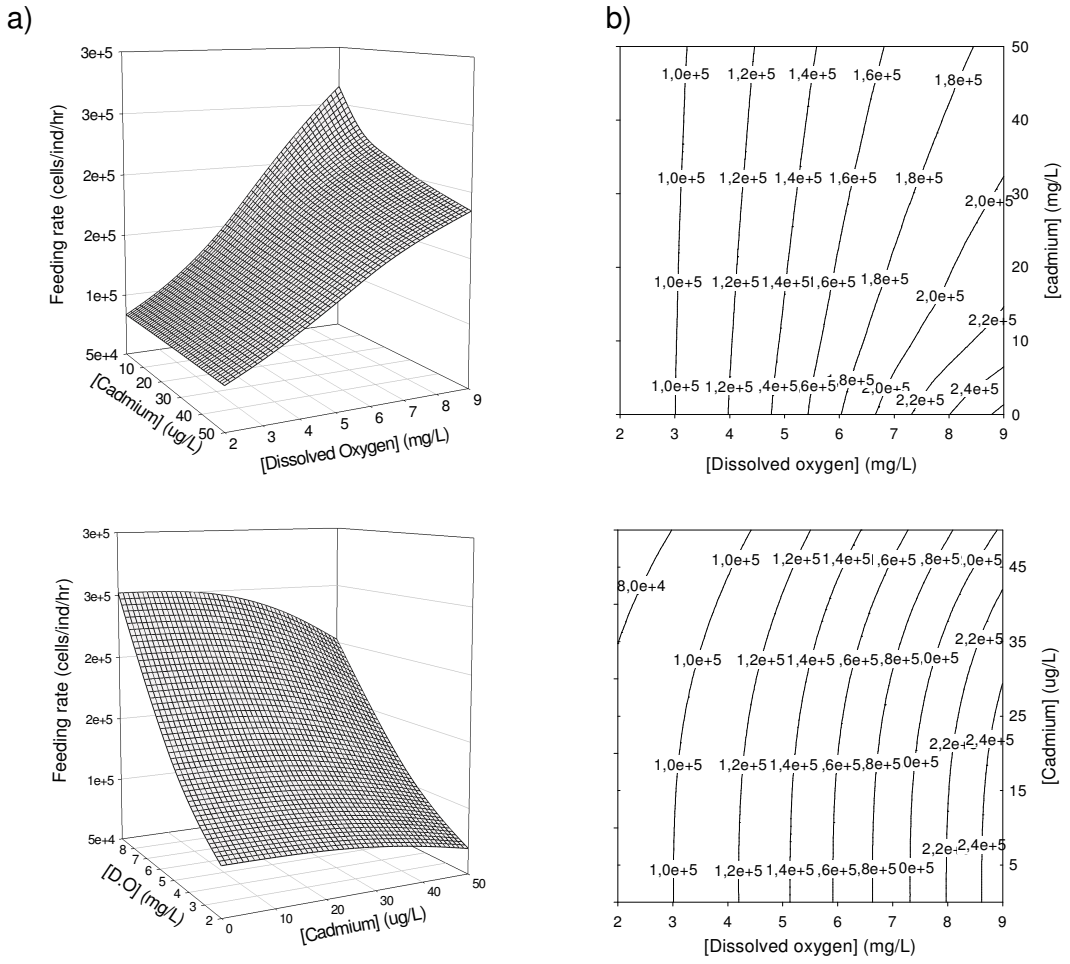

Fig.7 – Dose-response data from 24-hrs exposure for the combination of **Cd+D.O**., showing an antagonistic deviation from the CA model (upper row) and the IA model (lower row) for *Daphnia magna*'s feeding response: a) 3D mesh and b) 2D isobolic surfaces. Control value for D.O is 9mg/L.

Post-exposure data poorly fitted the CA model (SS=8.00×10^{10}, r^2= 0.603) presenting a significant antagonistic behaviour (χ^2 test, $p<0.05$; SS=6.76×10^{10}, a=1.45, r^2=0.665) as showed in fig. 8a) and 8b). Also without a good accuracy,

data was fitted to IA model (SS = 1.16×10^{11}, r^2=0.422) but no deviations were observed (p>0.05).

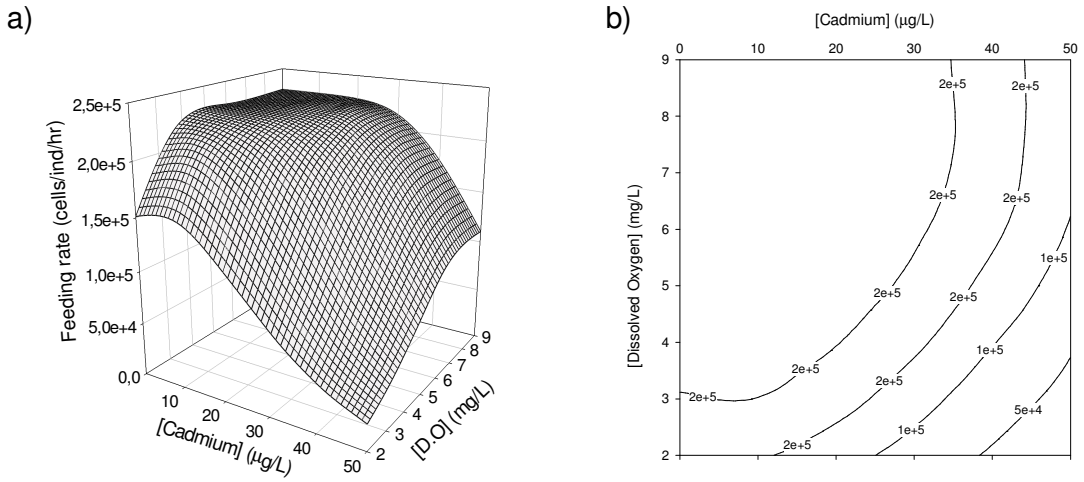

Fig.8 – Dose-response data from 4-hrs post-exposure for the combination of **Cd + D.O**, showing an antagonistic deviation from the CA model for *Daphnia magna*'s feeding response: a) 3D mesh and b) 2D isobolic surfaces. Control value for D.O is 9mg/L.

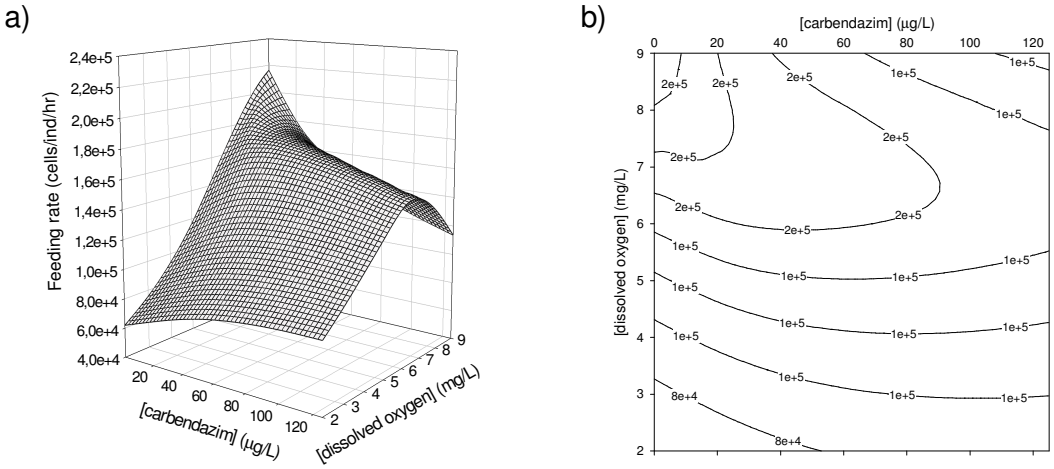

Fig.9 – Dose-response data from 24-hrs exposure for the combination of **CBZ + D.O**, showing an antagonistic deviation from the IA model: for *Daphnia magna*'s feeding response: a) 3D mesh and b) 2D isobolic surfaces. Control value for D.O is 9mg/L.

Data from 24-hrs feeding exposure to CBZ and DO combination fitted the IA model (SS=2.78×10^{10}, r^2=0.623) although not very accurately. When comparing to

the IA model, observed deviations for antagonism were significant (χ^2 test, $p<0.05$; $SS=1.62\times10^{10}$, $a=3.41$, $r^2=0.781$) (fig.9a and b).

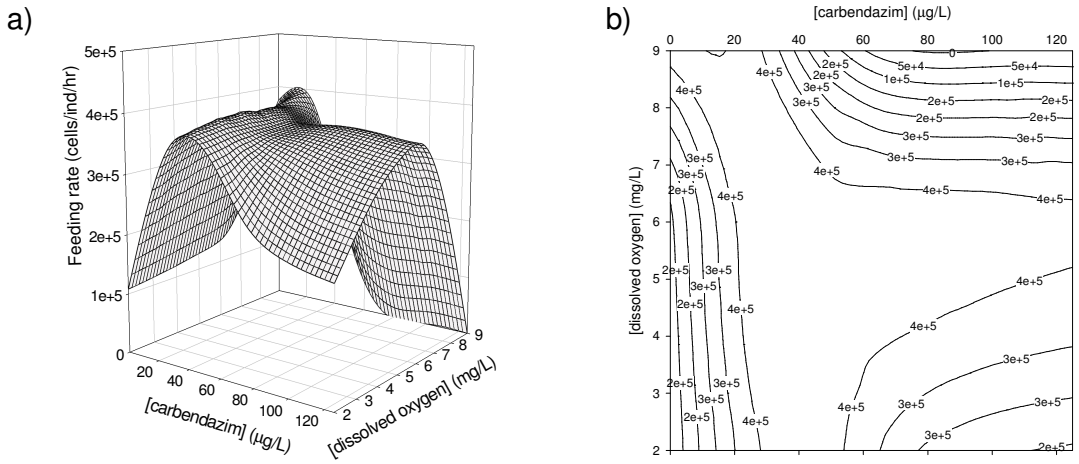

Fig.10 – Dose-response data from 4-hrs post-exposure for the combination of **CBZ + D.O**., illustrating an antagonistic deviation from the IA model. Representations of *Daphnia magna*'s feeding response: a) 3D mesh and b) 2D isobolic surfaces. Control value for D.O is 9mg/L.

Also for post-exposure data, a significant antagonistic behaviour was observed when compared to IA model (χ^2 test, $p<0.05$; $SS=1.96\times10^{11}$, $a=30.08$, $r^2=0.695$) (fig.10a and 10b).

Discussion

Single stressor exposures

In this study, the two conceptual models for predicting mixtures toxicity, CA and IA, based on the similarity or dissimilarity of the compounds mode of action (MoA) were tested. However, some chemicals can have ambiguous MoA, i.e. they may interact during uptake, distribution, metabolism and excretion processes (toxicokinetc phase), as well at the receptor level, cellular target and/or organ (toxicodynamics phase) (Cassee *et al*. 1998). So, in mixtures or combinations with one or more compounds revealing ambiguous MoA, both IA and CA models must be tested.

Carbendazim, as a benzimidazole, affect cell division by acting as mitotic spindle poison (European Medicines Agency 2004) while cadmium affects cells by elevating lipid peroxidation (Stohs and Bagchi 1995), a process initiated by reactive oxygen species (ROS), which causes the degradation of cells membrane (Rikans and Hornbrook 1997). Effects of low D.O levels are related with the process of induction of hemoglobin that occurs at hypoxia conditions (Gorr *et al*. 2004), autoxidation of the heme and consequent release of superoxide radicals involved in oxidative stress (Choi *et al.* 2000) that leads to severe injuries on cells viability (Jones 1985). Since cadmium and low dissolved oxygen concentrations affect cells by inducing oxidative stress, i.e. in a toxicodynamic approach exhibit a similar action, but however using different physiological mechanisms (dissimilar MoA by a toxicokinetic perspective) was decided to test both IA and CA models. As carbendazim MoA is related with mitotic inhibition process and consequently dissimilar from Cd and D.O modes of action, only IA model was tested when CBZ was present in mixture.

The LC50 value found for cadmium was in accordance with several studies with *Daphnia* sp.. Barata *et al.* (2000) studied the convergence of genotypic responses from lethal to sublethal exposures on daphnids and found a LC50 value for 48-hours that ranged from 30 to 219µg Cd/L. Ward and Robinson (2005) tested cadmium resistance in *D. magna* from eight different sources and obtained a LC50-48hrs value that ranged from 26 to 120µg Cd/L whereas, Shaw *et al.* (2006) in a comparative study of single and mixture toxicities of cadmium and zinc on several daphnids species observed a 48-hrs LC50 value for *D. magna* of 101µg Cd/L.

At the sublethal level, an increase on feeding inhibition was observed in our study for cadmium as in accordance to McWilliam and Baird (2002) study, however a different EC50 value of 1.31µg Cd/L was achieved by these authors.

Although producing a feeding depletion during Cd exposure, a dissimilar effect was observed in post-exposure period, since a significant increase on feeding rates was observed. This is an opposite result than those found by McWilliam and Baird (2002) where cadmium also caused a significant depletion after the exposure period.

For CBZ (as pure active ingredient) lethal and sublethal data are scarce, however several studies with similar chemical nature compounds (benzimidazoles) and commercial formulations were found. Oh *et al.* (2006) tested the toxicity of six benzimidazoles frequently used on veterinary activities and obtained 48-hrs LC50 values of 16.5 to 1,168µg/L for *Daphnia magna*. Benzimidazoles related compounds used by Oh *et al.* act as mitotic spindle poisons that impair cell division and mis-segregate chromosomes into the daughter cells, resulting in aneuploidy (European Medicines Agency 2004). Although with similar MoAs, these compounds showed differences in toxicity due to bioconcentration factor as related with different compounds lipophilicity. The 48hrs LC50 values of 110 and 350µg CBZ/L were also reported for *D. magna* by the Pesticide Ecotoxicity Database from U.S.EPA (2000a). Van Wijngaarden (1998) studied the toxicity of the commercial fungicide Derosal® (carbendazim as active ingredient) in several aquatic invertebrates and obtained a 48hrs LC50 value of 320µg/L for *D. magna*.

In our study, the feeding performance of daphnids for carbendazim exposure showed a significant inhibition at levels above 70µg CBZ/L for 24 hours with a 50% reduction near 100µg CBZ/L and again in compliance with the LOEC value for reproduction of 92.8µg CBZ/L found by Van Wijngaarden (1998). Recovery of daphnids feeding rates to levels near the control was observed during the post-exposure period and may indicate that these concentrations are not high enough to cause persistent effects.

The evaluation of the effects caused by different DO levels must be interpreted cautiously because decreasing concentrations of D.O implies an increase of stress. Data from exposure to extremely low concentrations of dissolved oxygen showed a switch between survival and mortality at a LC50 level of 0.5mg DO/L. Probably at lower DO levels (in this case, close to 0.5mg DO/L) an induction of haemoglobin synthesis occur (Gorr *et al.* 2004) in order to support aerobic metabolism, followed by an autoxidation of the heme group, leading to a release of superoxide radicals involved in oxidative stress processes. This was observed in a short-term period of 24-hrs by Choi *et al.* (2000) using chironomid larvae. Even for high levels of DO (above normoxia) oxidative stress may occur (Jones 1985) leading to severe injuries on cells viability. Nebeker *et al.* (1992) and

Sprague (1963) tested the resistance of four freshwater crustaceans to low oxygen and high temperature levels and obtained a 24-hrs LC50 value for *Hyalella azteca* of 0.7mg DO/L. Data from feeding exposures at different DO concentrations also showed a significant decrease in feeding rates, however less abrupt than in the acute test exposures. A possible reason may be the fact that at sublethal effect levels an increase in the activities of antioxidant enzymes (essentials on detoxification of reactive oxygen species) can occur (Pinto *et al.* 2003). This effect persisted after exposure to low DO levels probably due to the short-time of recovery for all metabolic processes affected by oxidative injuries.

Mixture exposures

The analysis of adverse effects of chemical mixtures or combinations with natural stressors comprised two main conceptual models based on the effect of individual compounds: concentration addition (CA) and independent action (IA). Similar or dissimilar MoAs can be linked to toxicokinetics or toxicodynamics phases since interactions may occur during uptake, distribution, metabolism and excretion or at the receptor level, cellular target and/or organ, respectively (Cassee *et al*. 1998).

Carbendazim is known to affect cell division by inhibiting β-tubulin assembly during mitosis, while cadmium affects cells in many ways: by elevating lipid peroxidation (Stohs and Bagchi 1995), a process initiated by reactive oxygen species (ROS), which causes the degradation of cells membrane (Rikans and Hornbrook 1997), or by causing DNA damages when in presence of hydrogen peroxide induced by oxidative stress (Badisa *et al.* 2007). Additionally, Cd also may indirectly generate free radicals by replacing copper and iron in various cytoplasm and membrane proteins leading to an increasing of unbound free Cu and Fe ions participating in oxidative stress *via* Fenton reactions (Casalino *et al*. 1997). So, based on all this evidences we concluded that since Cd and CBZ have different modes of action, only IA model should be tested.

At the toxicokinetic point of view, the IA model data showed that when cadmium is dominant, it will produce more severe effects. This might be explain by the important role of Cd in several processes that leads to disruption of cellular

homeostasis, such as oxidative stress (Pinto 2003), DNA damages (Badisa *et al.* 2007), membrane depolarization and acidification of the cytoplasm (Conner and Schimid 2003). These effects compared to those caused by carbendazim are more short-term effective when impairing cells viability than the long-term characteristic effects of carbendazim.

With sublethal doses exposure, Cd and carbendazim mixture revealed an antagonistic effect probably due to a reduction on cadmium toxicity caused by the induction of metallothioneins (MT), i.e. proteins of detoxification both in algae and *Daphnia* (Fraysse *et al.* 2006; Robinson 1989), by cells' defensive mechanisms. This induction of MT can also be linked to the presence of CBZ in mixture, since Mosleh and co-workers (2005) found that exposure to the fungicide fenhexamid increased metallothionein levels in *Tubifex tubifex.*

In the acute experiments, synergism observed at low DO and high Cd combined concentrations (after IA model fit) can be explained by the induction of haemoglobin in order to support aerobic metabolism (Gorr *et al.* 2004) and production of free radicals (Casalino *et al*. 1997), resulting both mechanisms in oxidative stress. Other reason might be the fact that at short-time exposure to hypoxia conditions, *Daphnia* increase heart and ventilation rates leading to a higher uptake of cadmium (Paul *et al*. 1998; Pirow *et al*. 2001).

The antagonistic effect that resulted from CA model fit to acute combination between Cd and low D.O levels was rejected by the calculation of the LC50 values for Cd (see table 3).

In the acute experiments, after IA model fit, synergism observed at low DO and high CBZ is probably related with the production of reactive oxygen species (ROS) by hypoxia conditions and consequently a reduction on cellular detoxification processes. This synergism was, in part, confirmed by the calculation of the LC50 values of CBZ at different DO levels (see table 3).

The synergistic behaviour verified by the decrease of the LC50 values as oxygen concentration decreases showed the higher accuracy of the deviations from the IA model prediction than those from the CA model for acute exposure between Cd and low DO levels.

At a sublethal exposure level, the antagonistic behaviour obtained, from both combined experiments, Cd+DO and CBZ+DO, was not supported by the calculation of the EC50 values at different DO levels, because these values showed a synergistic effect (see table 4). This means that neither CA, IA nor deviations functions models were accurate models to fit the obtained data.

The toxicity caused by the pre-exposure to the combination of Cd and low DO levels decreased, since an increase of the EC50 values from the 24-hrs to the 4-hrs period (see table 4) was observed. This can be interpreted as an apparent recovery in the feeding activity of the daphnids, probably caused by the increase of oxygen and the consequent re-stabilization of metabolic rates.

Conclusions

The first highlight that can be addressed from these results is that deviations from reference models were found in all combinations studied (synergism, antagonism and dose ratio dependency).

Other feature observed from our results analysis was the switch from synergism to antagonism revealed by almost IA deviations assessments, respectively from acute to sublethal exposures.

A different behaviour was observed for CA model fit where antagonistic deviations were verified for all mixtures, independently of the degree of exposure.

Since synergistic effects are associated to extreme environmental conditions, such as high temperatures and low DO levels, by directly and/or indirectly increasing the toxicity of chemicals presents in stressors combinations, as reported by several studies (Folt *et al*. 1999; Hanazato and Dodson 1995; Heugens *et al*. 2003; Heugens *et al*. 2006; Lewis and Horning 1991) we can conclude that deviations from IA model were the best functions to describe our acute exposures behaviour. This was also confirmed by the decrease of the LC50 values observed as DO levels decrease, i.e. at higher stress levels.

For sublethal exposures to DO levels the antagonistic deviation observed on both IA and CA models fits showed a failure on the prediction of combined toxicity, by underestimation.

Interpretation of mixtures behaviours from post-exposure assessments must be done carefully because this nested model as well as the IA/CA models and the different deviations concepts, are based on the direct toxicity caused by exposure to toxicants, while the post-exposure effects are reported to a pre-exposure period. However, this does not invalidate the use of this nested model to assess effects of a pre-exposure to combined stressors.

Toxicokinetics and toxicodynamics studies can contribute significantly to understand the main toxicological pathways behind a particular response of complex mixtures. The measure of the AChE activity and/or of the antioxidant enzymes such as, catalase, superoxide dismutase, glutathione peroxidase and GST, are examples of biomarkers that could help us to better understand toxicities behaviour.

Actual interpretation of combined effects is still difficult because chemicals and/or environmental stressors toxicity evaluation is not yet a routine in ecotoxicology resulting in a lack of data for comparison. However, a significant effort has been made in the last years, in order to invert this reality, as is example the EU project Nomiracle “Novel Methods for Integrated Risk assessment of Cumulative Stressors in Europe”.

Acknowledgments

The study was supported by the EU Integrated project NoMiracle (Novel Methods for Integrated Risk assessment of Cumulative Stressors in Europe; http://nomiracle.jrc.it) contract No. 003956 under the theme under the EU-theme "Global Change and Ecosystems" topic “Development of risk assessment methodologies”, coordinated by Dr. Hans Løkke at NERI, DK-8600 Silkeborg, Denmark.

References

Allen Y, Calow P, Baird DJ. 1995. A Mechanistic Model of Contaminant-Induced Feeding Inhibition in *Daphnia magna*. Environmental Toxicology and Chemistry 14(9):1625-1630.

Altenburger R, Backhaus T, Boedeker W, Faust M, Scholze M, Grimme LH. 2000. Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*:

Mixtures composed of similarly acting chemicals. Environmental Toxicology and Chemistry 19(9):2341-2347.

ASTM. 1980. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians, Report E-729-80. American Standards for Testing and Materials. Philadelphia, P.A.

Backhaus T, Altenburger R, Boedeker W, Faust M, Scholze M, Grimme LH. 2000. Predictability of the toxicity of a multiple mixture of dissimilary acting chemicals to *Vibrio fischeri*. Environmental Toxicology and Chemistry 19(9):2348-2356.

Badisa VLD, Latinwo LMO, Caroline O., Ikediobi CO, Badisa RB, Ayuk-Takem LT, Nwoga J, West J. 2007. Mechanism of DNA damage by cadmium and interplay of antioxidant enzymes and agents. Environmental Toxicology 22(2):144-151.

Baird DJ, Soares AMVM, Girling A, Barber I, Bradley MC, Calow P. 1989. The long-term maintenance of *Daphnia magna* Straus for use in ecotoxicity tests: problems and prospects. In Lokke, H., Tyle, H., Bro-Rasmussen, F. (Eds.). Proceedings of the First European Conference on Ecotoxicology. Lyngby, Denmark,:pp 144-148.

Barata C, Baird D, Nogueira A, Soares A, Riva M. 2006. Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. Aquatic Toxicology 78(1):1-14.

Barata C, Baird DJ. 2000. Determining the ecotoxicological mode of action of toxicants from measurements on individuals: results from short duration chronic tests with *Daphnia magna* Straus. Aquatic Toxicology 48(2-3):195-209.

Casalino E, Sblano C, Landriscina C. 1997. Enzyme activity alteration by cadmium administration to rats: The possibility of iron involvement in lipid peroxidation. Archives of Biochemistry and Biophysics 346(2):171-179.

Cassee FR, Groten JP, van Bladeren PJ, Feron VJ. 1998. Toxicological evaluation and risk assessment of chemical mixtures. Critical Reviews in Toxicology 28(1):73-101.

Chapman PM, Wang FY, Janssen CR, Goulet RR, Kamunde CN. 2003. Conducting ecological risk assessments of inorganic metals and metalloids: Current status. Human and Ecological Risk Assessment 9(4):641-697.

Choi JH, Roche H, Caquet T. 2000. Effects of physical (Hypoxia, Hyperoxia) and chemical (potassium dichromate, fenitrothion) stress on antioxidant enzyme

activities in *Chironomus riparius* Mg. (Diptera, Chironomidae) larvae: Potential biomarkers. Environmental Toxicology and Chemistry 19(2):495-500.

Conner SD, Schimid SL. 2003. Regulated portals of entry into the cell. Nature 422:37-44.

Connolly NM, Crossland MR, Pearson RG. 2004. Effect of low dissolved oxygen on survival, emergence, and drift of tropical stream macroinvertebrates. Journal of the North American Benthological Society 23(2):251-270.

Cuppen JGM, Van den Brink PJ, Camps E, Uil KF, Brock TCM. 2000. Impact of the fungicide carbendazim in freshwater microcosms. I. Water quality, breakdown of particulate organic matter and responses of macroinvertebrates. Aquatic Toxicology 48(2-3):233-250.

European Commission. 1999. Commission of the European Communities, Community Strategy for Endocrine Disruptors, a range of substances suspected of interfering with the hormone systems of humans and wildlife, COM 706 final.

European Commission. 2001. Directive 2455/2001/EC of the European Parliament and of the Council of 20 November 2001 establishing improvements on Directive 2000/60/EC. List of priority hazardous substances in the field of water policy. Official Journal of the European Communities. pL331/1-L331/5.

European Medicines Agency. 2004. Fenbendazole (extrapolation to alll ruminants) summary report (4). EMEA/MRL/866/03-Final.Committee for Medicinal Products for Veterinary Use. Veterinary Medicines and Inspections, London, UK.

Folt CL, Chen CY, Moore MV, Burnaford J. 1999. Synergism and antagonism among multiple stressors. Limnology and Oceanography 44(3):864-877.

Fraysse B, Geffard O, Berthet B, Queau H, Biagianti-Risbourg S, Geffard A. 2006. Importance of metallothioneins in the cadmium detoxification process in *Daphnia magna*. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 144(3):286-293.

Gorr T, Cahn J, Yamagata H, Bunn H. 2004. Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna*. Is hypoxia-inducible factor-dependent. The Journal of Biological Chemistry 279(20):36038-36047.

Hanazato T, Dodson SI. 1995. Synergistic, Effects of Low-Oxygen Concentration, Predator Kairomone, and a Pesticide on the Cladoceran *Daphnia pulex*. Limnology and Oceanography 40(4):700-709.

Haney JF. 1985. Regulation of Cladoceran Filtering Rates in Nature by Body Size, Food Concentration, and Diel Feeding Patterns. Limnology and Oceanography 30(2):397-411.

Heugens EHW, Kraak MHS, Admiraal W, Hendriks AJ, Jager T, Creyghton R, Van Straalen NM. 2003. Temperature-dependent effects of cadmium on *Daphnia magna*: accumulation versus sensitivity. Environ Sci Technol 37(10):2145-51.

Heugens EHW, Tokkie LTB, Kraak MHS, Hendriks AJ, van Straalen NM, Admiraal W. 2006. Population growth of *Daphnia magna* under multiple stress conditions: Joint effects of temperature, food, and cadmium. Environmental Toxicology and Chemistry 25(5):1399-1407.

International Agency for Research on Cancer of USA. 1997. Berylium, cadmium, mercury and exposures in the glass manufacturing industry. Summary of data reported and evaluation. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans.

Jak RG, Maas JL, Scholten MCT. 1996. Evaluation of laboratory derived toxic effect concentrations of a mixture of metals by testing fresh water plankton communities in enclosures. Water Research 30(5):1215-1227.

Jones DP. 1985. The role of oxygen concentration in oxidative stress: hypoxic and hyperoxic models. In Sies H. ed, Oxidative Stress. Academic, New York, NY, USA, pp:152-196.

Jonker MJ, Piskiewicz AM, Castella NII, Kammenga JE. 2004. Toxicity of binary mixtures of cadmium-copper and carbendazim-copper to the nematode *Caenorhabditis elegans*. Environ. Toxicology and Chemistry 23(6):1529-1537.

Jonker MJ, Svendsen C, Bedaux JJM, Bongers M, Kammenga JE. 2005. Significance testing of synergistic/antagonistic, dose-level dependent, or dose ratio-dependent effects in mixture dose response analysis. Environmental Toxicology and Chemistry 24(10):2701-2713.

Lewis PA, Horning WB. 1991. Differences in acute toxicity test-results of three reference toxicants on *Daphnia* at two temperatures. Environmental Toxicology and Chemistry 10(10):1351-1357.

McWilliam RA, Baird DJ. 2002. Postexposure feeding depression: A new toxicity endpoint for use in laboratory studies with *Daphnia magna*. Environmental Toxicology and Chemistry 21(6):1198-1205.

Minitab 2003. Minitab Incorporation Statistical Software, version Release 14.1.

Mosleh YY, Paris-Palacios S, Couderchet M, Biagianti-Risbourg S, Vernet G. 2005. Metallothionein induction, antioxidative responses, glycogen and growth changes in *Tubifex tubifex* (Oligochaete) exposed to the fungicide, fenhexamid. Environmental Pollution 135(1):73-82.

Nebeker AV, Onjukka ST, Stevens DG, Chapman GA, Dominguez SE. 1992. Effects of Low Dissolved-Oxygen on Survival, Growth and Reproduction of *Daphnia, Hyalella* and *Gammarus*. Environmental Toxicology and Chemistry 11(3):373-379.

Nogueira A.1996. Moddeling the physiological performance of *Daphnia* (Crustacea:Cladocera) under stress. PhD thesis. Stirling University, Stirling, UK.

Norwood WP, Borgmann U, Dixon DG, Wallace A. 2003. Effects of metal mixtures on aquatic biota: A review of observations and methods. Human and Ecological Risk Assessment 9(4):795-811.

OECD. 2004. OECD Guidelines for the Testing of Chemicals. Guideline 202: Daphnia sp., Acute Immobilisation Test, adopted April 2004.

Oh SJ, Park J, Lee MJ, Park SY, Lee JH, Choi K. 2006. Ecological hazard assessment of major veterinary benzimidazoles: Acute and chronic toxicities to aquatic microbes and invertebrates. Environmental Toxicology and Chemistry 25(8):2221-2226.

Olmstead AW, LeBlanc GA. 2005. Toxicity Assessment of Environmentally Relevant Pollutant Mixtures Using a Heuristic Model. Integrated Environmental Assessment and Management 1(2):114-122.

Paul RJ, Colmorgen M, Hüller S, Tyroller F, Zinkler D. 1997. Circulation and respiratory control in millimetre-sized animals (*Daphnia magna, Folsomia candida*?) studied by optical methods. Journal of Comparative Physiology B 167(6):399-408.

Paul RJ, Colmorgen M, Pirow R, Chen YH, Tsai MC. 1998. Systemic and metabolic responses in *Daphnia magna* to anoxia. Comparative Biochemistry and Physiology. a-Molecular & Integrative Physiology 120(3):519-530.

Pinto E, Sigaud-Kutner TCS, Leitao MAS, Okamoto OK, Morse D, Colepicolo P. 2003. Heavy metal-induced oxidative stress in algae. Journal of Phycology 39(6):1008-1018.

Pirow R, Baumer C, Paul RJ. 2001. Benefits of haemoglobin in the cladoceran crustacean *Daphnia magna*. Journal of Experimental Biology 204(20): 3425-3441.

Rand GM, Petrocelli SR. 1985. Fundamentals of aquatic toxicology: Methods and applications. United States: Hemisphere Publishing,New York, NY; FMC Corp., Princeton, NJ. Pages: 666 p.

Rikans LE, Hornbrook KR. 1997. Lipid peroxidation, antioxidant protection and aging. Biochimica Et Biophysica Acta-Molecular Basis of Disease 1362(2-3):116-127. Robinson N. 1989. Algal metallothioneins: secondary metabolites and proteins. Journal of Applied Phycology 1(1):5-18.

Shaw JR, Dempsey TD, Chen CY, Hamilton JW, Folt CL. 2006. Comparative toxicity of cadmium, Zinc, and mixtures of cadmium and zinc to daphnids. Environmental Toxicology and Chemistry 25(1):182-189.

Sprague JB. 1963. Resistance of 4 Freshwater Crustaceans to Lethal High Temperature and Low Oxygen. Journal of the Fisheries Research Board of Canada 20(2):387-415.

Stohs SJ, Bagchi D. 1995. Oxidative Mechanisms in the Toxicity of Metal-Ions. Free Radical Biology and Medicine 18(2):321-336.

Systat 2004. Systat Software, Incorporation. SigmaStat for Windows version 3.10.

Systat 2006. Systat Software, Incorporation. SigmaPlot for Windows version 10.0

Tomlin C. 1994. A World Compendium. The Pesticide Manual. Incorporation of the agrochemicals handbook. Cambridge: British Crop Protection Council p:1341 .

U.S.EPA. 2000a. Pesticide Ecotoxicity Database. EFFED - Environmental Fate and Effects Division. Washington, D.C.

U.S.EPA. 2000b. Supplementary guidance for conducting health risk assessment of chemical mixtures. Risk Assessment Forum EPA/630/R-00/002 Washington DC, USA.

van Wijngaarden RPA, Crum SJH, Decraene K, Hattink J, van Kammen A. 1998. Toxicity of Derosal (active ingredient carbendazim) to aquatic invertebrates. Chemosphere 37(4):673-683.

Wiggins PR, Frappell PB. 2000. The influence of haemoglobin on behavioural thermoregulation and oxygen consumption in *Daphnia carinata*. Physiological and Biochemical Zoology 73(2):153-160.

Wrona FJ, Prowse TD, Reist JD, Hobbie JE, Levesque LMJ, Macdonald RW, Vincent WF. 2006. Effects of ultraviolet radiation and contaminant-related stressors on Arctic freshwater ecosystems. Ambio 35(7):388-401.

IV.1. *Discussão*

Os dados obtidos na avaliação isolada de cada um dos stressores em estudo, estão de acordo com os vários estudos já efectuados (Heugens *et al*. 2003; Kersting and Vanwijngaarden 1992; Khangarot and Ray 1989; Nebeker *et al*. 1992; Shaw *et al*. 2006; U.S.EPA 2000a; van Wijngaarden *et al*. 1998), apresentando aumentos na toxicidade à medida que o factor de stress aumenta. A única excepção foi a estimulação da actividade alimentar em *Daphnia magna*, após um período de pré-exposição a concentrações subletais de cádmio. De facto, alguns estudos já efectuados (McWilliam and Baird 2002; Taylor *et al*. 1998) revelaram uma persistente inibição alimentar causada pela exposição ao cádmio após a mudança dos organismos para meio não contaminado.

Na avaliação da toxicidade aguda da mistura entre o cádmio (Cd) e o carbendazim, verificou-se um efeito mais severo quando o Cd é dominante na mistura. Várias razões podem estar por detrás deste facto, sendo uma delas a vasta diversidade de danos causados pelo cádmio tais como, stress oxidativo, danos no DNA, despolarização da membrana e acidificação do citoplasma (Badisa *et al*. 2007; Conner and Schimid 2003; Pinto *et al*. 2003) que, a curto prazo, podem ser irreversíveis na viabilidade celular.

Comparando os efeitos subletais resultantes da exposição às misturas Cd/carbendazim e Ni/clorpirifos, foram obtidas diferenças nas respostas. Na primeira mistura foi observado um efeito antagonista enquanto que na segunda um efeito sinergístico. Provavelmente, esta diferença estará relacionada com o facto de além de inibidor da acetilcolinesterase, o clorpirifos também induzir a baixas doses, a produção de espécies reactivas de oxigénio (ROS) (Jager et al. 2007) aumentando assim a toxicidade conjunta da mistura. A razão para o efeito antagonista resultante da combinação Cd/carbendazim tem a ver com a indução de metalotioninas (MT) presentes nas algas e nas dáfnias (Fraysse *et al*. 2006; Robinson 1989) como resultado dos mecanismos defensivos das células. Este processo de indução de MT foi também já observado em *Tubifex tubifex* pela presença de um outro fungicida (Mosleh *et al*. 2005).

Em relação aos efeitos agudos dos stressores naturais em combinação com os diferentes tóxicos, podémos observar que para todas as exposições a baixas e altas temperaturas, foram observados desvios ao modelo teórico baseado na acção independente dos compostos em mistura. Quanto aos efeitos subletais, apenas na combinação clorpirifos e altas temperaturas os dados tiveram um melhor ajuste com o modelo conceptual IA.

A mesma análise pode ser feita para a avaliação dos efeitos resultantes das misturas de cádmio e carbendazim e das combinações com níveis baixos de oxigénio dissolvido. Também neste caso foram observados desvios dos modelos conceptuais nas experiências agudas e crónicas.

Efeitos sinergísticos são muitas vezes associados a condições ambientais extremas, tais como temperaturas elevadas e níveis de oxigénio dissolvido baixos, uma vez que directa ou indirectamente aumentam a toxicidade dos tóxicos presentes em mistura (Folt *et al*. 1999; Heugens *et al*. 2003; Heugens *et al.* 2006; Lewis and Horning 1991). Um comportamento semelhante foi verificado neste estudo para exposições agudas a níquel e clorpirifos a temperaturas elevadas e de cádmio e carbendazim a concentrações baixas de oxigénio. Processos como o aumento da produção de hemoglobina (Lamkemeyer *et al*. 2003; Seidl *et al*. 2005) que induz a produção de espécies reactivas de oxigénio e consequentemente o stress oxidativo, ou o aumento do metabolismo (Viswanathan and Murti 1989) estão relacionados com os efeitos das altas temperaturas. Relativamente aos mecanismos associados às concentrações baixas de oxigénio, podemos salientar o aumento das taxas cardíacas e ventilatórias (Paul *et al*. 1998; Pirow *et al*. 2001) com a consequente entrada de maior quantidade de substância tóxica e a produção de hemoglobina (Gorr et al. 2004) de forma a manter o metabolismo aeróbio, levando uma vez mais à produção de stress oxidativo.

O comportamento antagonista revelado nas exposições subletais de carbendazim a níveis baixos de oxigénio dissolvido estará relacionado com o aumento das taxas metabólicas como resposta à redução de oxigénio. Este aumento não conduzirá a uma situação de stress oxidativo severo uma vez que as concentrações de oxigénio não são suficientemente baixas para que, em 24 horas, possa induzir a produção de espécies reactivas de oxigénio em larga

escala. No entanto, o aumento do metabolismo pode ser suficiente para estimular as actividades defensivas das células.

O antagonismo obtido para a combinação de cádmio a concentrações baixas de oxigénio, após o ajuste aos modelos de acção independente (AI) e adição de concentração (AC), foi rejeitado pelo cálculo do EC50 para cada concentração de oxigénio. Assim podemos concluir que, o desvio aos modelos conceptuais de AC e AI mostrou falhas na previsão da toxicidade conjunta de cádmio e níveis baixos de oxigénio dissolvido.

Relativamente aos resultados obtidos para a exposição subletal de níquel e clorpirifos a temperaturas baixas e altas, podemos concluir que apenas na combinação de clorpirifos e temperaturas altas se verificou uma falha na previsão do modelo de acção independente, assim como as funções que descrevem os desvios já mencionados, já que através do cálculo do EC50 para cada temperatura, verificou-se que o efeito resultante foi mais severo a temperaturas mais altas (24ºC) e menos severo a temperaturas próximas de 20ºC. Este comportamento terá a ver com o facto de a temperaturas próximas de 24ºC a actividade alimentar da *Daphnia magna* atingir o seu máximo (Koh et al. 1997) e também pela típica produção de hemoglobina a temperaturas mais altas (Lamkemeyer et al. 2003). O efeito sinergístico observado para a exposição sublethal de clorpirifos a temperaturas próximas de 16ºC pode ser explicado pelo facto da diminuição da temperatura conduzir ao aumento da sensibilidade do organismo tendo em conta parâmetros subletais (Smit and VanGestel 1997).

O sinergismo obtido a partir da combinação de concentrações que provocam efeitos subletais de níquel e temperaturas altas era esperado e justificado pela provável indução do processo de stress oxidativo (Lamkemeyer *et al*. 2003; Seidl *et al*. 2005). O efeito sinergístico obtido pelas combinações de baixas concentrações de níquel e temperaturas baixas, não foi totalmente confirmado, pois os cálculos dos valores de EC50 relativos à taxa de alimentação a 18ºC e a 16ºC, apresentaram uma diminuição na toxicidade conjunta da mistura (antagonismo).

A interpretação dos comportamentos das misturas referentes ao período de pós-exposição deve ser efectuada com cautela, uma vez que o modelo

matemático utilizado, bem como os conceitos de adição de concentração e acção independente, são baseados na toxicidade directa causada pela exposição a stressores, algo que não acontece no período de pós-exposição, onde os efeitos se reportam ao período de pré-exposição.

IV.2. *Conclusões gerais*

Uma das primeiras conclusões a tirar deste trabalho tem a ver com o facto de na maioria das misturas/combinações testadas, os desvios aos modelos teóricos de adição de concentração (AC) e acção independente (AI), se terem verificado. Este estudo vem demonstrar a variedade de efeitos e comportamentos que podem resultar da combinação de tóxicos/stressores bastante comuns no nosso ambiente, uma vez que todos os possíveis desvios aos modelos ocorreram (sinergismo, antagonismo, dependência da dose ou do rácio da mistura).

Outra característica observada a partir da análise destes resultados foi a alteração de efeitos sinergísticos para efeitos antagonistas, de exposições letais para subletais, respectivamente, verificada para praticamente todas as misturas ajustadas ao modelo de AI.

Um comportamento diferente foi o observado para a avaliação de misturas/combinações entre cádmio, carbendazim e concentrações baixas de oxigénio. De facto, quando ajustados os dados ao modelo de adição de concentração, o efeito resultante das misturas foi sempre de menor toxicidade, ou seja, antagonista, o que não estava de acordo com o observado.

Uma vez que os efeitos sinergísticos estão normalmente associados a condições ambientais extremas como temperaturas altas e concentrações baixas de oxigénio dissolvido, podemos concluir que os desvios obtidos a partir do modelo de AI foram os que melhor previram o comportamento agudo dessas combinações.

O insecticida clorpirifos e o factor temperatura mostraram ser os principais factores na indução de efeitos mais severos, ao nível das exposições agudas, enquanto que o níquel exerceu esse papel nas exposições subletais.

A validação a partir de estudos toxicocinéticos e toxicodinâmicos parece ser de primordial importância para este tipo de avaliações, uma vez que permitirá compreender os principais processos fisiológicos envolvidos na complexidade das misturas. O estudo de biomarcadores como a actividade da acetilcolinesterase, ou aqueles relacionados com o processo de stress oxidativo como a catalase (CAT) ou a glutationa S-transferase (GST) podem também ajudar a responder a algumas das dúvidas aqui levantadas.

Finalmente, podemos concluir que o modelo matemático aqui utilizado e anteriormente descrito por Jonker *et al* (2005) mostrou ser, uma boa ferramenta na avaliação de respostas dadas pelos dafnídeos expostos a combinações binárias.

Este estudo pretende ser uma pequena contribuição na nova abordagem da avaliação conjunta do risco de saúde humana e do risco ecológico, baseada nos efeitos cumulativos da exposição combinada a stressores múltiplos.

IV.3. *Referências bibliográficas*

Badisa VLD, Latinwo LMO, Caroline O., Ikediobi CO, Badisa RB, Ayuk-Takem LT, Nwoga J, West J. 2007. Mechanism of DNA damage by cadmium and interplay of antioxidant enzymes and agents. Environmental Toxicology 22(2):144-151.

Conner SD, Schimid SL. 2003. Regulated portals of entry into the cell. Nature 422:37-44.

Folt CL, Chen CY, Moore MV, Burnaford J. 1999. Synergism and antagonism among multiple stressors. Limnology and Oceanography 44(3):864-877.

Fraysse B, Geffard O, Berthet B, Queau H, Biagianti-Risbourg S, Geffard A. 2006. Importance of metallothioneins in the cadmium detoxification process in *Daphnia magna*. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 144(3):286-293.

Gorr T, Cahn J, Yamagata H, Bunn H. 2004. Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna*. Is hypoxia-inducible factor-dependent. The Journal of Biological Chemistry 279(20):36038-36047.

Heugens EHW, Kraak MHS, Admiraal W, Hendriks AJ, Jager T, Creyghton R, Van Straalen NM. 2003. Temperature-dependent effects of cadmium on

Daphnia magna: accumulation versus sensitivity. Environ Sci Technol 37(10):2145-51.

Heugens EHW, Tokkie LTB, Kraak MHS, Hendriks AJ, van Straalen NM, Admiraal W. 2006. Population growth of *Daphnia magna* under multiple stress conditions: Joint effects of temperature, food, and cadmium. Environmental Toxicology and Chemistry 25(5):1399-1407.

Jager T, Crommentuijn T, Van Gestel CAM, Kooijman SALM. 2007. Chronic exposure to chlorpyrifos reveals two modes of action in the springtail *Folsomia candida*. Environ. Pollut. 145:452-458.

Jonker MJ, Svendsen C, Bedaux JJM, Bongers M, Kammenga JE. 2005. Significance testing of synergistic/antagonistic, dose-level dependent, or dose ratio-dependent effects in mixture dose response analysis. Environmental Toxicology and Chemistry 24(10):2701-2713.

Kersting K, Vanwijngaarden R. 1992. Effects of Chlorpyrifos on a Microecosystem. Environmental Toxicology and Chemistry 11(3):365-372.

Khangarot BS, Ray PK. 1989. Investigation of Correlation between Physicochemical Properties of Metals and Their Toxicity to the Water Flea *Daphnia magna* Straus. Ecotoxicology and Environmental Safety 18(2):109-120.

Koh HL, Hallam TG, Lee HL. 1997. Combined effects of environmental and chemical stressors on a model *Daphnia* population. Ecological Modelling 103(1):19-32.

Lamkemeyer T, Zeis B, Paul RJ. 2003. Temperature acclimation influences temperature-related behaviour as well as oxygen-transport physiology and biochemistry in the water flea *Daphnia magna*. Canadian Journal of Zoology-Revue Canadienne De Zoologie 81(2):237-249.

Lewis PA, Horning WB. 1991. Differences in acute toxicity test-results of three reference toxicants on *Daphnia* at two temperatures. Environmental Toxicology and Chemistry 10(10):1351-1357.

Mosleh YY, Paris-Palacios S, Couderchet M, Biagianti-Risbourg S, Vernet G. 2005. Metallothionein induction, antioxidative responses, glycogen and growth changes in *Tubifex tubifex* (Oligochaete) exposed to the fungicide, fenhexamid. Environmental Pollution 135(1):73-82.

McWilliam RA, Baird DJ. 2002. Postexposure feeding depression: A new toxicity endpoint for use in laboratory studies with Daphnia magna. Environmental Toxicology and Chemistry 21(6):1198-1205.

Nebeker AV, Onjukka ST, Stevens DG, Chapman GA, Dominguez SE. 1992. Effects of Low Dissolved-Oxygen on Survival, Growth and Reproduction of *Daphnia*, *Hyalella* and *Gammarus*. Environmental Toxicology and Chemistry 11(3):373-379.

Paul RJ, Colmorgen M, Pirow R, Chen YH, Tsai MC. 1998. Systemic and metabolic responses in *Daphnia magna* to anoxia. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 120(3):519-530.

Pinto E, Sigaud-Kutner TCS, Leitao MAS, Okamoto OK, Morse D, Colepicolo P. 2003. Heavy metal-induced oxidative stress in algae. Journal of Phycology 39(6):1008-1018.

Pirow R, Baumer C, Paul RJ. 2001. Benefits of haemoglobin in the cladoceran crustacean *Daphnia magna*. Journal of Experimental Biology 204(20):3425-3441.

Robinson N. 1989. Algal metallothioneins: secondary metabolites and proteins. Journal of Applied Phycology 1(1):5-18.

Seidl M, Pirow R, Paul R. 2005. Acclimation of the microcrustacean *Daphnia magna* to warm temperatures is dependent on haemoglobin expression. Journal of Thermal Biology 30(7):532-544.

Shaw JR, Dempsey TD, Chen CY, Hamilton JW, Folt CL. 2006. Comparative toxicity of cadmium, zinc and mixtures of cadmium and zinc to daphnids. Environmental Toxicology and Chemistry 25(1):182-189.

Smit CE, VanGestel CAM. 1997. Influence of temperature on the regulation and toxicity of zinc in *Folsomia candida* (Collembola). Ecotoxicology and Environmental Safety 37(3):213-222.

Taylor G, Baird DJ, Soares AMVM. 1998. Surface binding of contaminants by algae: consequences for lethal toxicity and feeding to Daphnia magna Straus. Environ. Toxicol. Chem. 17:412 - 419.

U.S.EPA. 2000a. Pesticide Ecotoxicity Database. EFFED - Environmental Fate and Effects Division. Washington, D.C.

van Wijngaarden RPA, Crum SJH, Decraene K, Hattink J, van Kammen A. 1998. Toxicity of Derosal (active ingredient carbendazim) to aquatic invertebrates. Chemosphere 37(4):673-683.

Viswanathan PN, Murti CRK. 1989. Effects of temperature and humidity on ecotoxicology of chemicals - In: Bourdeau P. (ed.), Haines J.A. (ed.), Klein W. (ed.), Krishna Murti C.R. (ed.) Ecotoxicology and climate. New York: J. Wiley. p: 139-154.