

***Daphnia magna bioassays to detect novel
Eco-toxicological effects of priority and emergent
contaminants.***

PhD Dissertation

Bruno Campos

Director Dr. Carlos Barata

Acknowledgments

First of all I have to acknowledge the Ministerio de Economía y Ciencia for my scholarship (**BES-2009-022741** associated to the research project **CGL2008-0189**), CSIC and IDAEA for the infrastructure and administrative support to develop this work.

I also need to thank the UFZ, Leipzig; Germany and the University of Stockholm, Sweden, for the opportunity of learn new techniques and develop parts of this work in their facilities.

In the end of this 4 years trip, I have to thank to so many people that I would most surely miss someone, so I will make it short.

I want and I need to thank Carlos Barata, my director, for giving me the opportunity of working with him, for all the guidance, the discussions and for teaching me so much. It was a real pleasure to work with you. I also want to thank Benjamin Piña, my un-official co-director, who was always available to help and elucidate me with his tremendous knowledge; this thesis would've not been the same without you.

I want to thank to everyone that worked with me, I have learned so much from so many amazing people I have met. It was a real pleasure to have met you all; I am a better person because of you.

Finally I need to thank to my family, my mother, my father, my brothers for being my safe house, even from far away, and for putting up with my bad moods and temper. I owe you so much that I will never be able to repay. This is also yours.

It is common to say that a PhD is a road that changes you professionally and personally. To me this is so very true. Besides giving me knowledge, along the road I met my other half, my better half. I hope I can be for you what you have been for me every day. Grazie ☺

It was a roller coaster ride, with so many ups and downs, but worth every moment.

THANK YOU.

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Summary

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Assessing the risks of emerging pollutants requires an understanding of both the exposure regime and the effects of the chemical on different organisms. There is increasing evidence that the presence of many emerging pollutants in aquatic ecosystems may have detrimental effects on aquatic biota. Of special concern are those emerging pollutants that may act as putative endocrine disrupters in non-vertebrate species, causing unexpected effects that may pose a threat to the ecosystem sustainability, but also others that may act as chemosensitizers, i.e., by blocking active trans-membrane transporters, impairing detoxification mechanisms. Other contaminants, like engineered nanoparticles, are recently raising awareness among researchers, due to their small size, below 100nm, and aggregation properties. Several reviews claim that new toxicity procedures should be used to evaluate nanomaterial toxicity due to their unique size characteristics that for example may aggregate and sequestrate other contaminants or natural particles.

In my studies, I used *Daphnia magna*, a historical and widely used test organism, to assess ecotoxicological effects of chemicals in freshwater.

In chapters 2, 3 and 4, each one published as an original study (Campos et al., 2013a; Campos et al., 2012a; Campos et al., 2012b), I assessed the effects of two pharmaceuticals belonging to the group of “Selective Serotonin Reuptake Inhibitors”: fluoxetine and fluvoxamine, which are widely used to treat human depression, and the detergent 4-nonylphenol. These three chemicals have the property of enhancing offspring production in *D. magna* at low environmental levels. In order to understand, the Mode of Action (MoA) of these two very different families of chemicals several experiments were performed. Both the SSRIs and 4-nonylphenol increased offspring production and SSRIs also increased the juvenile developmental rates at limiting but not at high food ration levels. Factorial life-history experiments and reproductive assays showed that exposure to SSRIs increased juvenile development rate, clutch size, and decrease offspring size at low and intermediate levels of food rations. Such life-history changes were interpreted as a shift towards higher food levels, that is,

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SSRIs change the perception of food levels on exposed *D. magna* individuals making them to behave like they grew at higher food levels. Enhanced reproductive effects of SSRIs were reversed by the presence of the 5-HT antagonist cyproheptadine, indicating that in *D. magna* SSRIs targeted serotonin metabolic paths like in humans. Respirometry and survival assays and biochemical analyses of lipids, proteins and carbohydrate levels showed that adult females exposure to SSRIs increased oxygen consumption rates and decreased carbohydrate levels. These changes did not affect survival under starving conditions, but they significantly affected the capacity of the exposed animals to survive under anoxic conditions.

Factorial life-history experiments and reproductive assays showed that low exposure levels of 4-nonylphenol increased offspring number but decreased offspring size and at higher exposure levels impaired severely reproduction. These responses have detrimental fitness effects since smaller offspring takes longer to mature and reproduce and having less offspring directly affects population growth rates.

The analysis of the *D. magna* transcriptome showed that serotonin metabolism, neuronal developmental processes, and carbohydrates and lipid metabolism pathways appeared as selectively affected by SSRIs treatment, whereas 4-nonylphenol deregulated genes from the carbohydrate metabolism and the ecdysone regulatory pathway. These changes in physiological and metabolic pathways are consistent with previously reported SSRI and 4-nonylphenol hormetic effects in *D. magna*. SSRIs show a MoA consistent with its pharmacological effect in humans while 4-nonylphenol effect seems to be performed through changes in the ecdysone regulatory pathways.

In chapter 5, published in Campos et al. (2013b). I evaluated the extent to which different forms of nano-TiO₂ aggregate with microalgae, decreasing food levels and consequently growth and reproduction of *D. magna* individuals. To accomplish my objective, I performed different experiments using three different types of nano-TiO₂ differing in their coating or crystalline structure but of similar primary size (20nm) plus a micron-sized bulk material, two nano-TiO₂ exposure levels (1 and 10mg/L) and different food levels that included non-limiting and limiting food conditions. Effects were assessed using three different types of

assays. Assays included chronic reproduction 21-day tests using semi-static conditions that followed OECD protocols, but also custom designed tests performed in 1L bottles holding larger water columns. The latter included two exposure scenarios: semi-static and re-suspension of algae. Effects on *D. magna* were determined from growth and reproduction performance measurements.

Results indicated that the high ion levels in culture medium lead to the aggregation of nanoparticles followed by particle destabilization. Nanoparticle aggregates interacted with the algae cells, forming clusters. Large TiO₂-algae agglomerates settled readily, dramatically depleting the concentration of available food for *Daphnia*. At limiting food rations, depletion of food by nanoparticle aggregation had dramatic effects on reproduction and fitness of exposed *D. magna* at 1mg/L irrespectively of the particle form. At high food rations effects were only observed for one of the nano-TiO₂, P-25, at high exposure levels (10mg/L) under both semi-static and particle re-suspension conditions, which suggest that P-25 effects were mediated by clogging the gut and hence diminishing food acquisition. These results indicate that nano-TiO₂ may affect the transfer of energy throughout the planktonic aquatic food webs increasing the settlement of edible particles from the water column.

In chapter 6, submitted to Aquatic Toxicology, I attempted to characterize the presence and activity of the multixenobiotic resistance proteins (MXR) in *D. magna*. This system has already been studied in several aquatic organisms, mainly bivalve species, whereas there is only a *in silico* study on *D. pulex*, a close relative of *D. magna*. I sequenced new partial sequences of four genes, coding for full transporters and studied their transcriptional pattern across life-stages and with juveniles exposed to MXR inducers. All the genes were transcribed early in embryos and some of them were induced in juvenile stages by pentachlorophenol and mercury. Associated efflux activity was monitored using three fluorescent substrate dyes, which are specific of different mammalian transporter types, combined with specific MXR transporter inhibitors (chemosensitizers). Dye accumulation assays indicated that MXR is functional in juveniles and induced by pentachlorophenol, mercury and dacthal. Toxicity bioassays performed with model toxic substrates of ABCB1 (mitoxantrone) and

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ABCC (chlorambucil) transporters applied in combination with the studied chemosensitizers showed joint toxic effects greater than additivity in most cases. This means that the MXR system in *D. magna* is active and plays an important role detoxifying contaminants.

Published papers in this thesis:

Campos, B., Garcia-Reyero, N., Rivetti, C., Escalon, L., Habib, T., Tauler, R., Tsakovski, S., Piña, B., Barata, C., 2013a. Identification of Metabolic Pathways in *Daphnia magna* Explaining Hormetic Effects of Selective Serotonin Reuptake Inhibitors and 4-Nonylphenol Using Transcriptomic and Phenotypic Responses. **Environmental Science & technology** **47**, 9434-9443.

Campos, B., Piña, B., Carlos, B.C., 2012a. Mechanisms of action of selective serotonin reuptake inhibitors in *Daphnia magna*. **Environmental Science and Technology** **46**, 2943-2950.

Campos, B., Piña, B., Fernández-Sanjuán, M., Lacorte, S., Barata, C., 2012b. Enhanced offspring production in *Daphnia magna* clones exposed to serotonin reuptake inhibitors and 4-nonylphenol. Stage- and food-dependent effects. **Aquatic Toxicology** **109**, 100-110.

Campos, B., Rivetti, C., Rosenkranz, P., Navas, J.M., Barata, C., 2013b. Effects of nanoparticles of TiO₂ on food depletion and life-history responses of *Daphnia magna*. **Aquatic Toxicology** **130-131**, 174-183.

CHAPTER 1

General Introduction

1. Background

"Sustainable development is development that meets the needs of the present without compromising the ability of future generations to meet their own needs."

When the first green wave appeared in the mid and late 1960s, it was considered a feasible task to solve pollution problems. The visible problems were mostly limited to point sources, well defined, and a comprehensive environmental technology was available. It was even seriously discussed in some of the most industrialised and developed countries what was called "zero discharge" could be achieved (Kümmerer, 2010). Nevertheless, it became clear in the early 1970s that zero discharge would be too expensive and would possibly have an impact in society that was not acceptable.

Meanwhile, it has been shown that what can be called "the environmental crisis" is much more complex than we initially thought.

In 1987 the United Nations released a report, written under the auspices of the General Assembly, which was to serve as a keystone on the way how our society should manage the new challenges that were arising. This was known as the Brundtland Report, "Our common future" (World Commission on Environment and Development, 1987) and it highlighted the way how we should manage our society, by establishing beacon's or objectives, that would allow us to keep our development needs while keeping in mind the needs of future generations, and taking into account economics, politics, engineering, culture and environmental concerns.

According to the same report, Sustainable development and the way how it was defined contains within it two key concepts:

- the concept of 'needs', in particular the essential needs of the world's poor, to which overriding priority should be given; and
- the idea of limitations imposed by the state of technology and social organization on the environment's ability to meet present and future needs.

As at this moment there are more than 72 million organic and inorganic substances registered (CAS 2013), and more than 43 million are commercially available for purchase and usage. This shows us how we are a “chemical” society and surrounded at all moments by natural and synthetic substances, which may threaten the environment due to their more or less toxic effects on plants, animals, humans and entire ecosystems. In most industrialised countries comprehensive environmental legislation was introduced to regulate the wide spectrum of different pollution sources. Trillions of dollars have been invested in pollution abatement on a global scale, but it seems that two or more new problems emerge for each problem that is solved (Mitsch and Jørgensen, 2003).

1.1. How to deal with large number of chemicals surrounding us

More than 100,000 chemicals have been introduced in the 20th century alone, that are used in our everyday life, either in households, industries or agriculture. We have “blindly” introduced these chemicals without realising the consequences for the environment and directly and indirectly for human health (Hartung, 2011). The EU started to list these chemicals in the late 1970s, and since the mid-1980s it has been compulsory to set up an environmental risk assessment (ERA) for all new chemicals. The original idea was to make environmental risk assessments for all the chemicals already in use, but it is going very slowly, and at the present rate, we shall not be able finish ERAs for all the applied chemicals in the next decade. Probably, it is necessary to speed up the evaluation of the chemicals in use, for instance by forging a closer cooperation between the environmental agencies and the chemical industry, in order to obtain a realistic picture of the environmental risk associated with the many chemicals we apply today. In order to achieve this, the EU started the Registration, Evaluation, Authorisation and Restriction of Chemical substances. (REACH). REACH is the European Community Regulation on chemicals and their safe use (EC 1907/2006). The law entered into force on 1 June 2007. The REACH Regulation places greater responsibility on industry to manage the risks from chemicals and to provide safety information on the substances. Manufacturers and importers are required to gather information on the

properties of their chemical substances, which will allow their safe handling, and to register the information in a central database.

One of the main reasons for developing and adopting the REACH Regulation was the large number of substances that have been manufactured and placed on the market in Europe for many years, sometimes in very high amounts, and yet there is insufficient information on the hazards that they pose to human health and the environment.

This makes urgent to fill these information gaps to ensure that industry is able to assess hazards and risks of the substances, and to identify and implement the risk management measures to protect humans and the environment and when possible to proceed to a gradual substitution of the most hazardous substances by others with less impact, on human health, but also on wildlife and ecosystems.

1.2. Aquatic Ecosystems protection

The aquatic ecosystem is one of the most affected natural systems by chemicals. Compounds that are used in industry, agriculture or household, have a high probability to end in ground waters, rivers, lakes, freshwater catchments, estuaries, reservoirs and marine waters.

Many of the most used substances have been submitted to ERA, with a highlight on potential effects on aquatic species and have been organized in hazard lists according to their hazardous potential. One of the most important lists is the Priority substances, which were defined within the EU Water Frame Directive (WFD) (EU Directive 2000/60/EC). The WFD objectives are to reduce and/or eliminate the potential emission sources of the catalogued chemicals and remove them from the environment. By making use of the available information on the effects and occurrence of chemicals, the priority chemicals list was proposed by the EU, taking into account the expert judgment of scientific committees and research programs and following the recommendations of other European political and civil institutions.

This list included originally 33 priority substances and 8 other pollutants (Decision 2455/2001/EC). More recently it was proposed to increase this list with another 15 additional priority substances, 6 of them designated as priority

hazardous substances. But also to add other considerations about some other chemicals:

- the introduction of biota standards (Environmental Quality Standards, (EQS) for several substances;
- provisions to improve the efficiency of monitoring and the clarity of reporting with regard to certain substances behaving as ubiquitous, persistent, bio accumulative and toxic (PBT) substances;
- provision for a watch-list mechanism designed to allow targeted EU-wide monitoring of substances of possible concern to support the prioritisation process in future reviews of the priority substances list.

This approach is useful, but unfortunately left out a great number of chemicals that are raising concerns, as the standard ERA evaluations do not cover specific sublethal effects that may have high impacts at ecological levels.

1.3. Emerging contaminants

In the last decades there has been a major development in analytical chemistry technologies, allowing scientists to detect chemicals in the range of the low part *per* trillion, or nanogram *per litre* (ng/L) and even picogram/L (pg/L). With these increased capabilities, we have now overwhelming evidence that “new” xenobiotic substances have been produced over the last decades and are nowadays ubiquitous in the aquatic environment. These substances, referred to as “emerging pollutants”, include a wide array of different compounds (as well as metabolites and transformation products). These “new” environmentally relevant chemicals are now often referred as Emerging Contaminants or contaminants of Emerging Concern (EC). (Barceló, 2003)), defined emerging pollutants as:” pollutants that are not yet regulated but may be candidates for future regulation depending on research, on their potential health effects and monitoring data regarding their occurrence in ecosystems, and since their high transformation or removal rates can be compensated by their continuous introduction into the environment, they do not need to be persistent in the environment to be present in the ecosystems”.

Broadly, they can be described also, as any synthetic or naturally occurring chemical that is not commonly monitored in the environment, but has been recently detected and is now raising awareness.

Emerging contaminants are not actually new. They can be present in the environment for decades, but only now we have the technology that allows us to detect them. Nevertheless, due to their large numbers, only few of these compounds are toxicologically evaluated, and most of them superficially. In view of that, the scientific community is becoming aware of the risks of ECs, and it is without surprise that these chemicals are now recollecting most of the resources for research, together with the priority chemicals for an in-depth ERA.

Nevertheless, it should be kept in mind that there is a big difference between priority and emerging chemicals. The former are or may pose a serious risk both for human populations or the environment, either by the high concentration levels or persistence. They are mostly well characterized and because they are mainly used in big volumes in industry and agriculture, they have well defined points of entrance to the environment and are somehow easy to track. ECs represent a shift in the traditional way how we face chemical pollution, being that many are industrially produced, they are incorporated in daily life products and their dispersion into the environment is diffuse, from domestic, commercial, industrial and agricultural runoffs and adequate data do not exist to determine their risks (Drewes and Shore, 2001; Younos, 2005).

Our ability to detect ECs in the environment may often exceed our ability to understand their risks. Initial studies of EC occurrence focused on locations where they were expected to occur, such as those downstream from intense urbanization or livestock production (Kolpin *et al.*, 2002). Several research documents show that ECs are present in the environment on a global scale and can occur in relatively undeveloped areas (Ashton *et al.*, 2004; Bendz *et al.*, 2005).

Are some ECs potentially harmful to ecological or human health? How do we allocate the scarce funds to study or control exposure to ECs when risks are poorly defined? Are there safe limits of exposure or ingestion? How do we respond to new compounds and new modes of action? These are some key questions that need answers (Glassmeyer, 2007).

Because many ECs are designed molecules, the diversity of effects that can be caused is very broad. Some ECs can act as endocrine disrupting chemicals (EDCs) and be a serious threat to the environment. EDCs have the potential to affect hormonal driven processes, such as reproduction and aquatic organisms are particularly susceptible to EDCs, because their entire life cycles are spent in continuous contact with these substances in water. Some ECs (hormones and pharmaceuticals) are produced to have biological effects and hence may have effects on non-target organisms at very low (ppb-ppt) levels.

Emerging Contaminants include the following wide range of substances including pharmaceuticals and personal care products (PPCPs), illicit drugs and drug of abuse, hormones and steroids, benzothiazoles, benzotriazoles, polychlorinated naphthalenes (PCNs), perfluorochemicals (PFCs), polychlorinated alkanes (PCAs), polydimethylsiloxanes (PDMSs), synthetic musks, quaternary ammonium compounds (QACs), bisphenol A (BPA), triclosan (TCS), triclocarban (TCC), as well as polar pesticides, veterinary products, industrial compounds/by-products, food additives and engineered nano-materials (Lapworth *et al.*, 2012).

For most emerging contaminants, there is currently little information regarding their potential toxicological significance in ecosystems particularly regarding effects from long-term, low-level environmental exposures.

There are thousands of chemicals and substances available for industrial processes and consumer goods, with additional chemicals being developed every day. Because many of these chemicals are relatively new, the effects of many on human health and the environment are not known.

The ever increasing need to conserve and reuse water can lead to increases in the number and concentration of many ECs and underlines the need for more scientific assessment to better understand the sources of ECs, the effectiveness of removal by wastewater and drinking-water-treatment processes, the transport and environmental fate of these compounds, and their potential for physiological effects on humans and wildlife. New scientific evidence about the exposure pathways and potential impacts of some of these compounds on human or environmental health is regularly being published (Claessens *et al.*, 2013; Cristale *et al.*, 2013; Drewes *et al.*, 2005; Gibbs *et al.*,

2007; Hutchinson *et al.*, 2013; Kinney *et al.*, 2006; Veldhoen *et al.*, 2006; Woodling *et al.*, 2006). They are products that we regularly use (or create) in our homes, businesses, farms and industry and yet adequate data are lacking to determine their actual risk (Hutchinson, 2007; Soin and Smagghe, 2007; Younos, 2005). Recent news headlines have declared potential human health and ecological concerns regarding the occurrence of personal care products and pharmaceuticals in the environment and the society is slowly becoming aware of the problems that we may face, due to a heavy consumption and use of ECs and disregarded disposal of them.

Due to the high number of substances that can be classified within ECs and the very different characteristics that they present, we will focus in this work in three classes:

Pharmaceuticals and Personal Care Products (PPCPs)

Alkylphenols (4-Nonylphenol)

Engineered Nano-Materials

Musks and chemosensitizer chemicals

1.3.1. Pharmaceuticals and personal care products (PPCPs)

In our daily life we use a high number of products, which make our life easier and/or better and more comfortable. Either it's the shampoo, toothpaste, deodorant, medicines or even our clothes; we are using hundreds of different chemical components that will then be disposed with our garbage or wastewater.

As long as pharmaceuticals and personal care products (PPCPs) have been used, they have been present in the environment. However, many of them are becoming persistent in the environment, due to the fact that as they degrade, more are added in a continuous way and our ever increasing use of PPCPs.

The consumption of pharmaceuticals is increasing in European countries, as in the rest of the world. It is estimated that the consumption of pharmaceuticals will increase at a 3-4% rate in weight per year (Daughton, 2004), and some specific groups of compounds may even be higher. Studies performed in OECD countries showed that antidepressants consumption increased by more than 200% from 2000 to 2007 (OECD, 2012) even though recent reports show a

significant delay of this increase in the last years, possibly due to the economic crisis and government budget cuts in health. Then again, the use of other personal care products are increasing even more, with the rise of emerging economies and millions of new consumers having access to consumer products. Due to persistence and increasing levels of many PPCPs, scientists are beginning to notice harmful effects in the environment.

PPCPs are molecules designed to have low cellular toxicity, and very specific effects and because of that the normal toxicological evaluation according to the guidelines, will more often than not, underestimate the effects on ecosystem level. Often, similar target biomolecules are present in non-mammalian organisms and so PPCPs can have adverse effects across species. Unless more is known about possible chronic effects of individual pharmaceuticals and other personal care products, conclusions concerning hazard potential or risks to the aquatic ecosystem are premature.

PPCPs can induce unexpected effects in non-mammalian organisms based on the difference in pharmacokinetics and pharmacodynamics, important parameters for occurring species. Disturbances of the reproductive and hormonal system, immune depression, neurobehavioral changes just to name some key targets, may have far reaching effects at the population level. This has become evident for endocrine disrupters such as steroid hormones used in contraceptives resulting in important adverse effects at environmentally relevant concentrations (Jobling *et al.*, 1998; Länge *et al.*, 2001; Parrott and Blunt, 2005). Pharmaceuticals can reach water bodies through sewage systems, industrial discharges, effluents from sewage treatment plants (STPs), aquaculture and livestock farming. Pharmaceuticals include hundreds of substances which are very different regarding physical-chemical properties and environmental behaviour.

Presently, pharmaceuticals can be detected in surface waters at the levels of $\mu\text{g/L}$ to mg/L and some are considered ubiquitous (Oosterhuis *et al.*, 2013). Nevertheless, their presence in the aquatic environment and impact on aquatic biota has not yet been studied adequately. Experimental evidence indicates that pharmaceuticals may cause harmful effects, such as morphological, metabolic

and sex alterations on aquatic species, induction of antibiotic resistance in aquatic pathogenic microorganisms, and disruption of biodegradation activities in STPs (Bottini *et al.*, 2010). Risk assessment studies and evaluations are in progress and new information is brought to attention every day. Nevertheless, the available scientific data are consistent with the introduction of some pharmaceutical indicators in extensive water monitoring to better define their actual impact on aquatic organisms and humans. Under these perspectives, the inclusion of emerging pharmaceuticals in the revision of EU List of Priority Substances under the Water Framework Directive 2000/60/EC should be implemented as well as the definition of respective environmental quality standards.

Nevertheless, there is a general lack of chronic toxicity data of PPCPs on non-target species and specially, pharmaceuticals need more investigation about potential long-term ecotoxicological effects, particularly with respect to potential disturbances in hormonal homeostasis (endocrine disruption), reproductive outputs, immunological status, gene activation and/or silencing during long-term, low doses exposure.

In-depth understanding of possible effects needs a mechanism-based approach focused on target molecules should yield more meaningful results and insights than traditional acute toxicity testing. Current data on acute and chronic toxicity of pharmaceuticals support to the conclusion that more target, or mode-of-action based investigations, will allow more relevant insights into effects on survival, growth and reproduction than traditional standard ecotoxicity testing. A useful new tool can be the Adverse Outcome Pathways (AOP) analysis, a conceptual analysis that tries to correlate effects and mode-of-action (Ankley *et al.*, 2010).

Current tests cover only a small set of laboratory organisms, and are often not sensitive enough thus not able to unravel adverse effects of pharmaceuticals due to their design. As a consequence, more specific tests are needed. Only chronic toxicity investigations using more specific toxicity parameters will lead to a more meaningful ecological risk assessment. When evaluating the effects of PPCPs on non-target species, we should keep in mind that, by design, these are biologically active molecules, that may or may not

have similar effects in non-target species, as many species share the structure of the target, due to evolutionary conservation. But they can also have unexpected effects in other organisms due to biological and physiological differences that will alter the pharmacodynamics and/or pharmacokinetics.

The first pharmaceuticals molecules were detected in the environment in the 1980's (Richardson and Bowron, 1985; Waggott, 1981). However, concerns over the presence and effects of this medical substances and their metabolites, increased in the last years of the 20th century. After the publication of the first reviews on the emerging concerns and the state of the art of PPCPs in freshwater systems (Daughton and Ternes, 1999; Fent *et al.*, 2006; Halling-Sørensen *et al.*, 1998; Heberer, 2002), the scientific community started directing their efforts towards the characterization of hazard potential of these substances.

Pharmaceuticals are designed to target specific metabolic and molecular pathways in humans and animals, but they often have important side effects. When introduced into the environment they may affect the same pathways in animals having identical or similar target organs, tissues, cells or biomolecules. Certain receptors in lower animals resemble those in humans, others however, are different or lacking, which means that dissimilar modes of actions may occur in lower animals or provoke unexpected effects due to biological differences and organization throughout all the levels of life on earth. It is important in this respect to recognize that for many drugs, their specific modes of actions are not well known and often not only one, but many different modes of actions occur simultaneously. Among other reasons, this makes specific toxicity analysis in lower animals difficult to perform. Despite this, toxicity experiments should be targeted and designed for specific targets of the pharmaceutical even in lower vertebrates and invertebrates, based on the hypothesis of similarity of modes of actions. However, current toxicity testing is not designed in this way, rather general and established test systems and traditional organisms according to guidelines are being used and traditional end points such as mortality are assessed (Fent *et al.*, 2006).

Pharmaceuticals are assessed for their acute toxicity by traditional standard tests according to established guidelines (e.g. OECD, U.S. EPA, ISO) using

established laboratory organisms such as algae, zooplankton and other invertebrates and fish. Acute toxicity data of pharmaceuticals were compiled by (Halling-Sørensen *et al.*, 1998) and (Webb, 2001), making a list of about 100 human pharmaceuticals from different sources. In the attempt to compare the different classes of pharmaceuticals in terms of acute toxicity, (Webb, 2001) noted that the most toxic classes were antidepressants, antibacterial and antipsychotics, but the range of responses within each of these categories was large, typically several orders of magnitude, and did not take into the equation the sublethal effects that can impair the ecosystem. (Heckmann *et al.*, 2007) studied the effects of ibuprofen on *Daphnia magna*. The previous authors showed that even though *D. magna* had a high tolerance to acute doses of Ibuprofen, offspring production was impaired at much lower doses. This drug, reduces female fertility by affecting the eicosanoid pathway, its intended pharmacological target in humans, thus, leading to a decrease of the population and a high impact at population level (Heckmann *et al.*, 2008b). This is a clear example of an unexpected effect of a pharmaceutical.

1.3.2. Anti-depressants

Anti-depressants consumption in human society is growing. The new generation anti-depressants are a group of molecules known as Selective Serotonin Reuptake inhibitors (SSRIs).

SSRIs are a widely used antidepressant class, which acts by inhibiting the re-uptake of serotonin (Hyttel, 1994). The neurotransmitter serotonin is involved in many mechanisms, both hormonal and neuronal, and it is also important in functions such as food intake and sexual behaviour. A pump directs serotonin from the synaptic space back to the pre-synapse, and SSRIs inhibit this pump, thus increasing the serotonin level in the synaptic cleft. Serotonin as a neurotransmitter occurs in vertebrates and invertebrates (Fong, 1998), however, the effects associated with this transmitter are different, and so are the effects of SSRIs. The role of serotonin in reproduction varies between different phyla. Besides having important functions as a neurotransmitter, serotonin may directly act on the immune system, alters appetite, influences behaviour and modulates sexual function, altering the nervous and associated hormonal systems. Serotonin mediates, among other processes, endocrine

functions in aquatic organisms such as parturition in fingernail clams *Sphaerium striatinum* (Fong *et al.*, 1998) and egg production, rate of fertilization and spawning in Japanese medaka *Oryzias latipes* (Foran *et al.*, 2004). SSRIs, by affecting the concentration of serotonin in the synaptic cleft, has been shown to have several effects already described in different phyla.

The SSRIs (fluoxetine and fluvoxamine) led to induction of parturition in fingernail clams and an induction of spawning in zebra mussels at concentrations as low as 32 ng/L (Fong, 1998; Fong *et al.*, 1998). More recently it was found that fluoxetine reduced the number of oocytes and spermatozoa of zebra mussel gonads at concentrations of 10ng/L (Lazzara *et al.*, 2012). Serotonin may stimulate ecdysteroids, ecdysone and juvenile hormone, responsible for controlling oogenesis and vitellogenesis (Nation, 2002), thus, it is expected that SSRIs may have an effect also in arthropod species. Indeed SSRIs are known to increase reproduction in *D. magna* and *C. dubia* at low doses (Flaherty *et al.*, 2001), and reduced them at high doses. An evaluation of five SSRIs (fluoxetine, fluvoxamine, paroxetine, citalopram, sertraline) showed negative effects on *C. dubia* reproduction by reduction of the number of neonates or brood per female after 7–8 days of exposure(Henry *et al.*, 2004).

Fluoxetine, the active compound of PROZAC, has been detected in sewage and stream water at concentrations of 12ng/L (Kolpin *et al.*, 2002) and 99ng/L (Metcalfe *et al.*, 2003) respectively. Fluoxetine maximal measure concentrations (100ng/L) were near the LOEC for zooplankton and other benthic organisms, so the margin of safety is very thin, and chronic effects at this point cannot be disregarded, in particular when the combined effects of pharmaceutical mixtures are taken into account. It should be noted, however, that more experimental data on chronic toxicity and on the bioaccumulation potential is needed to fully judge the environmental risk posed by individual pharmaceuticals.

1.3.3. Alkylphenols

Alkylphenols (APs) are starting materials for the production of many surfactants and antioxidants stabilizers used in commercial products like detergents, plastics and textiles. Namely, APs are used in the synthesis of alkylphenolethoxylates (APEs) and are also generated by the decomposition of

these substances in the environment. APEs are one of the most widely used classes of non-ionic surfactants in domestic and industrial products (Shang *et al.*, 1999). Among them, the most representative are nonylphenols (NPs), with a C9-alkyl group and octylphenols (OPs), with a C8-alkyl group. NP is the most commercially prevalent of the APs family, representing approximately 85% of the APs market, is present in many household products, even though it has been banned in Europe.

Long-chain APEs are susceptible to metabolism in natural environment, leading to the formation of APEs with short ethoxylate chain length (typically 1-3) such as APs and carboxylated APEs, which are more toxic and persistent than their precursor (Ahel *et al.*, 1994). The environmental fate of these metabolites is of significant interest because of their potential endocrine disrupting effects (Bennie *et al.*, 1998). For instance, NP ethoxylates are easily transformed to their main metabolite, NP, under anaerobic conditions, which is approximately 10 times more toxic than its ethoxylate precursor (Renner, 1997) and is known to disrupt normal hormonal function (Dachs *et al.*, 1999; Johnson and Sumpter, 2001). Of all APEs metabolites, OP is the strongest xeno-estrogen and can induce significant effects in fish at concentrations of 3 µg/L (Jobling *et al.*, 1996). On the other hand, NP is active at concentrations of 8 µg/L (Harris *et al.*, 2001).

Since 1998, 4-NP and 4-OP are listed by OSPAR as chemicals for priority action (OSPAR-Commission, 2011) and both APs are present in the Water Framework Directive (Directive 2000/60/EC) of substances and substance groups prioritized for action to achieve good quality surface water (Toyama *et al.*, 2010a; Toyama *et al.*, 2010b). The aim of these regulations is to ensure that emissions and losses of these substances to surface waters must be reduced to zero within the next two decades.

Whereas APs are worldwide distributed, the concentration detected in waters are geographic and legislation dependant. Water policy as well as water treatment facilities play an important role in the residues detected in each site. Furthermore, the monitoring of APs is basically restricted to OP and NP, and very little information is available on the fate of these common APs in the water.

APs were monitored in rivers and estuaries in England and Wales during 1993 (Blackburn and Waldock, 1995). Dissolved NP (and total extractable NP) ranged from 0.2 (0.5) to 53 (180) µg/L. Concentrations of NP were one order of magnitude lower in estuaries than in rivers, reflecting dispersion and dilution processes (Blackburn and Waldock, 1995). (Matthiessen and Law, 2002) reviewed the biological effects of contaminants in British estuaries and coastal waters for the last 100 years. Until 1970, the major pollution impacts on estuarine organisms was probably attributed to poorly treated sewage and oxygen depletion, whereas since the installation of wastewater treatment plants (WWTPs), the release of toxic micro-pollutants became a serious environmental problem and NP was identified in Tyne and Tees estuaries as contributing to toxic effects in the copepod *Tisbe battagliai*.

In the NE Spain, Wastewater Treatment Plants (WWTP) and river outflows were identified as a major source of organic micro-pollutants to coastal waters. Among other contaminants, OP, NP and NP monoethoxylated were identified as the major contaminants with concentrations from 0.013 to 0.061 µg/L for OP and NP in river water and 0.395 and 1.021 µg/L for OP and NP in wastewater effluents, respectively (Sánchez-Avila *et al.*, 2012).

Toxicological information on NPs is extensive but mainly focused on estrogenic and/or mammalian effects. The most abundant and studied compound is 4-n-NP. There is a general consensus among regulators that NP is estrogenic and hence it should be banned. Nevertheless, it is of paramount importance to determine the detrimental effects on multiple aquatic species. NP is metabolized in the liver and its metabolites excreted in the bile, mainly as glucuronide conjugates. However, extraction of faecal samples revealed that faeces mainly contained NP itself (Green *et al.*, 2003; Zalko *et al.*, 2003).

NP is considered a weak xeno-estrogen around 100 times less potent than 17-β-estradiol (Routledge and Sumpter, 1997). This means that it has the potential to be estrogenic but at high concentrations. Thus, it is not surprising to find that in rats, NP impaired first and second generation reproductive traits in males at doses of 150 mg/kg/day, which are quite high compared to those of 17-β-estradiol (0.1 mg/kg/day) (Chapin *et al.*, 1999; Nagao *et al.*, 2001; Tyl *et al.*, 2006). In the above mentioned studies systemic toxicity, however, occurred at lower doses (15 mg/kg/day), which also indicates that probably the dominant

mode of action of NP to mammals and ultimately to humans is systemic and not estrogenic.

Environmental health effects of NP in non-mammalian species have also been extensively studied in the aquatic environment. Like most APs, NPs are relatively soluble in water and are present in surface waters at quite high concentrations when compared with other pollutants. Concentrations of NP of 4.35 μ g/L have been measured in some coastal areas and Mediterranean rivers (Petrovic *et al.*, 2002a; Petrovic *et al.*, 2002b). Fish should also be susceptible to estrogenic effects by NP. There is experimental evidence that NP may cause estrogenic related effects (increase plasma/liver vitellogenin levels in males, impair male reproduction, alter sex characteristics and mating behaviour) on fish between 10 and 100 μ g/L in long term exposures but there is also evidence that NP impair non estrogenic endpoints such as growth and reproduction of fish at the same range. Thus again, it is not clear in the above reported studies if NP effects are systemic or truly estrogenic. Indeed, new evidence indicates that NP may interact with the lipidogenic pathways and act as an obesogen (Hao *et al.*, 2012).

In river locations, downstream discharges of effluents from WWTPs located nearby Barcelona city, *in vitro* estrogenic tests have identified NP as the most estrogenic compound present in surface waters (Céspedes *et al.*, 2004; Quirós *et al.*, 2005). This result contradicts those reported earlier in waste water effluents from England (Desbrow *et al.*, 1998) that found that estrogenic effects on rivers, downstream WWTP effluents were caused by steroid hormones like estradiol. The above mentioned discrepancies, however, could be explained by the fact that in most cases Spanish treatment plants have tertiary treatments that remove hormones whereas those of UK do not. These results have also been extrapolated in the field in the Llobregat river (NE, Spain) where authors reported high levels of vitellogenin in males collected after waste water plant effluent discharges (Solé *et al.*, 2000). Nevertheless, again, it is difficult to establish a close link between *in vitro* test and *in vivo* responses due to the co-existence in the field of many chemicals that can be estrogenic as well. A piece of evidence that may support the weakness of the estrogenic arguments in fish is the fact that today despite that NP is still present at high amounts in certain

parts of rivers within Spain there are not clear reported effects in fish (González *et al.*, 2012).

In aquatic invertebrates, NP appears to be quite toxic to small crustaceans at around 10µg/L. The mechanism of action of NP to aquatic invertebrates is unclear. According to (Barata *et al.*, 2004), most studies that have reported endocrine disruption effects of NP to aquatic invertebrates misinterpreted the results since observed inhibitory effects on growth and reproduction could be associated with feeding impairment, which is a general toxicity mechanism quite common across pollutants. Nevertheless, there is experimental data that indicates that NP at high exposure levels (> 100 µg/L) may affect the metabolism of ecdysteroids impairing embryo survival (LeBlanc *et al.*, 2000a).

Information on pollutants able to disrupt reproduction in an unexpected way, enhancing reproduction rather than inhibit it, are scarce and contradictory. Enhanced offspring production in *Daphnia* females exposed to pollutants has been reported for fluoxetine, 4-nonylphenol and dispersogen A (Brooks *et al.*, 2003a; Brooks *et al.*, 2003b; Flaherty and Dodson, 2005; Hammers-Wirtz and Ratte, 2000; LeBlanc *et al.*, 2000b), although inhibitory effects on reproduction have also been found for the first two chemicals. 4-Nonylphenol, has been reported having a similar effect, increasing reproduction. Indeed recent studies indicate that 4-nonylphenol may inhibit, un-change or enhance offspring production in *D. magna* (Brooks *et al.*, 2003a; Brooks *et al.*, 2003b; Flaherty and Dodson, 2005; Hammers-Wirtz and Ratte, 2000; LeBlanc *et al.*, 2000b).

1.3.4. Engineered Nanomaterials

Manufactured nanomaterials (MNMs) are a relatively new class of elemental metals, chemical compounds, and engineered materials with particle sizes in the nanometer range (<100 nm). Several classes of MNMs are now globally manufactured in hundreds to thousands of metric tons per year. These include MNMs for structural applications (ceramics, catalysts, films and coatings, and composite metals), skin care products (metal oxides), information and communication technologies (nanoelectronic and optoelectronic materials, organic light emitters, and nanophosphors), biotechnology (drug delivery,

diagnostic markers, and biosensors) and environmental technologies (nanofiltration and membranes) (Borm and Berube, 2008).

Naturally occurring (geogenic) nanomagnetite exists in some bacteria, which use this mineral to sense the Earth's magnetic field (Faivre *et al.*, 2005). Primary geogenic nanoparticles also occur as aerosols from ocean spray (salts and sulfates), volcanic emissions (sulfate aerosols), forest fires (soot and elemental carbon, and polycyclic aromatic hydrocarbons or PAHs) (Lucas and Akimoto, 2007). Primary anthropogenic nanoparticles are similar in composition to geogenic nanoparticles, ranging from less than PM1 ($\leq 1,000 \text{ nm}$) to ultrafine particles (UFP) ($\leq 100 \text{ nm}$). Sulfate, hydrogensulfate, and nitrate nanoparticles are emitted to the atmosphere from industrial sources and power plants, and nanocarbons are emitted from internal combustion (primarily diesel) engines. Major MNM sources with potential impacts to air, soil, surface water and groundwater are from industrial production, including amorphous silica, carbon blacks and fullerenes, and titanium and zinc oxides(Borm and Kreyling, 2004; Lucas and Akimoto, 2007).

MNMs is one of the fastest growing industries and it was estimated to be worth a trillion US Dollars in the near future (Nel *et al.*, 2006). Nanotechnology has the potential to deliver considerable benefits to the society, but at the same time, nanomaterials are at the centre of the discussion about risks of possible releases of very small particles in the environment and how can affect human health.

It is widely accepted that there is a need to analyse the risks associated with nanotechnology in order for nanotechnology and nanomaterials to be developed responsibly. Despite that manufactured nanomaterials are covered by the Directive 67/548/EEC, 793/93 and REACH, there is an unclear situation regarding requirements for risk assessment. The traditional risk assessment methodology has not yet been applied to nanoparticles, and there are no official guidelines on what constitutes an appropriate testing regimen. Another point is that the regulation of REACH, based on tons, needs to consider that there are many more nanoparticles in one ton than would be the case for larger particles. With regard to effects, there is insufficient data available to identify any generic rules governing the likely toxicology and ecotoxicology of nanoparticles. A new sub-discipline of toxicology, nanotoxicology, has emerged with the aim of

addressing the effects that may be associated with nanosized materials. There is also an urgent need for harmonized terminology and nomenclature for defining the physical characteristics of nanoparticles and their general properties. The measurement of specific properties and exposure levels is necessary. For those objectives, methods that measure physicochemical properties and that reliably detect nanoparticles in the environment are needed. There is a serious lack of information about relevant monitoring studies focused on the occurrence in the environment. Analytical methodologies are now beginning to be applied for the detection of nanoparticles in environmental samples. Nanotechnology is becoming an important policy area in the EU. The first step has been given by the Seventh Framework Program for research, which set aside an indicative budget for research into nanosciences and nanotechnologies and financed several European projects on an integrated perspective, clustered together under the 7th Framework Program of the EU (<http://www.nanosafetycluster.eu/>).

The study of the ecotoxicity of manufactured nanomaterials to aquatic organisms has a relative recent history when compared with that of other toxic substances (Baun *et al.*, 2008). Several reviews claim that new toxicity procedures should be used to evaluate MNM toxicity due to their unique characteristics of size being below 100 nm, the extremely high ratio surface/volume and their inherent toxicity (Handy *et al.*, 2012; Handy *et al.*, 2008a; Handy *et al.*, 2008b). At present, most studies have been focused in determining the bio-availability of MNM and their direct toxicity to cells, aquatic invertebrates and fish (Baun *et al.*, 2008). Fewer ones, however, have addressed indirect effects that MNM may have in aquatic biota such as in water column invertebrates due to their aggregation properties in aqueous solutions (Li *et al.*, 2011), that can lead to interactions with microscopic (phytoplankton, zooplankton) food particles. Although colloidal particles of geochemical and biological origin are abundant in the aquatic environment, unknown toxicological effects especially of engineered nanoparticles on organisms require comprehensive multi-disciplinary investigations. Numerous toxicological studies with nanomaterials had been conducted (Powers *et al.*, 2013; Sun *et al.*, 2013). The results of these eco-toxicological studies, however, are not consistent.

There is reported evidence that MNM like those of titanium dioxide (nano-TiO₂), fullerenes (C₆₀), or single walled carbon nanotubes aggregate to microalgae, copepods and *Daphnia* cell surfaces or carapaces, impairing their growth or swimming behaviour (Baun *et al.*, 2008; Li *et al.*, 2011; Metzler *et al.*, 2011; Oberdörster *et al.*, 2006).

Most nano-toxicity studies followed conventional protocols designed to test dissolved chemical species that may not be totally applicable to nanoparticles (Handy *et al.*, 2012). For example, many MNM form aggregates in water, thus experimental protocols designs to test toxicity effects of dissolved chemical products may not be valid.

1.4. Multidrug Resistance system (MDR)

More than four decades ago oncologists started realizing that many tumours presented an inherent resistance pattern, while other tumours were able to develop resistance through the time of treatments, against many of the chemotherapeutic drugs. This phenotype was described as being a multidrug-resistance pattern. After these findings, scientist from over the globe have established that this protection mechanism is due to the presence in the plasmatic membrane of transporter proteins that actively expel various cytotoxic agents from cancer cells, thus allowing the tumours to have an overtime resistance to chemotherapeutic treatments. This mechanism of resistance was named Multidrug/Multixenobiotic Resistance mechanism (Dano, 1973; Sarkadi *et al.*, 2006). In the following decades, researchers have been trying to clarify the mechanism behind this resistance and nowadays the role of these transporters is well defined both pharmacologically and in cell biology literature.

Multixenobiotic Resistance transporters belong to the evolutionary highly conserved superfamily of the ATP Binding Cassette (ABC) proteins, present in almost all living organisms, from prokaryotes to mammals (Sarkadi *et al.*, 2006), which suggests they are of ancestral origins. For example, *in silico* studies of several genomes, have identified many putative transporters, including 48 ABC transporters in the human genome, 41 in *Danio rerio* (Dean and Annilo, 2005a), 64 in the *Daphnia pulex* (Sturm *et al.*, 2009) and 65 in the sea urchin *Strongylocentrotus purpuratus* (Sodergren *et al.*, 2006).

In addition to Multidrug/Multixenobiotic transporters, ABC proteins also include ion channels, regulators of ion channels, receptors and proteins with roles in ribosome assembly and translation (Dean and Allikmets, 2001; Dean and Annilo, 2005b; Gottesman and Ambudkar, 2001). According to their domain architecture and sequence, metazoan ABC transporters are divided into subfamilies. The most important ones belong to seven subfamilies named ABCA to ABCG (Klein *et al.*, 1999).

ABC transporters are large membrane bound proteins, with specific and highly conserved nucleotide binding domains, one or two (NBDs,) which include two signatures, the ABC signature motif and the Walker B domain (Sturm *et al.*, 2009). The presence of these two signature sequences allows for the identification of the NBDs through all the families. It's in the NBDs that occurs the hydrolysis of ATP, releasing the energy that is needed for substrate transport, enabling conformational changes of the transporter, thus allowing for the efflux of the xenobiotics and/or metabolites out of the cells (Ramachandra *et al.*, 1998). Apart from the NBDs, the functional operation of ABC transporters also requires two or three trans-membrane domains (TMDs), where is located the substrate binding site. All eukaryotic ABC proteins are, either, full transporters combining all the required domains (2 NBDs and 2/3 TMDs) in one polypeptide, or half transporters consisting of 1TMD and one NBD that need to form homo- or heterodimers to be functional (Sturm *et al.*, 2009).

Out of the seven ABC families, three of them are directly involved in the efflux of xenobiotics and/or metabolites and thus they are the most relevant for toxicity studies. These are: the ABCB family, also known as P-glycoprotein (P-gp); the ABCC or Multidrug Resistance Protein (MRP) and the ABCG or Breast Cancer Resistance Protein (BCRP) (Figure 1.1)

Earlier studies associated MXR transporter activity only to the P-gp (ABCB) transporter proteins, however, recent studies have shown that MRP and BCRP (ABCG2) transporter proteins are also directly involved in the multidrug resistance mechanism. These proteins are expressed in tissues with important role for absorption, i.e. lung and gut, but also elimination and metabolism, like kidney and liver (Leslie *et al.*, 2005).

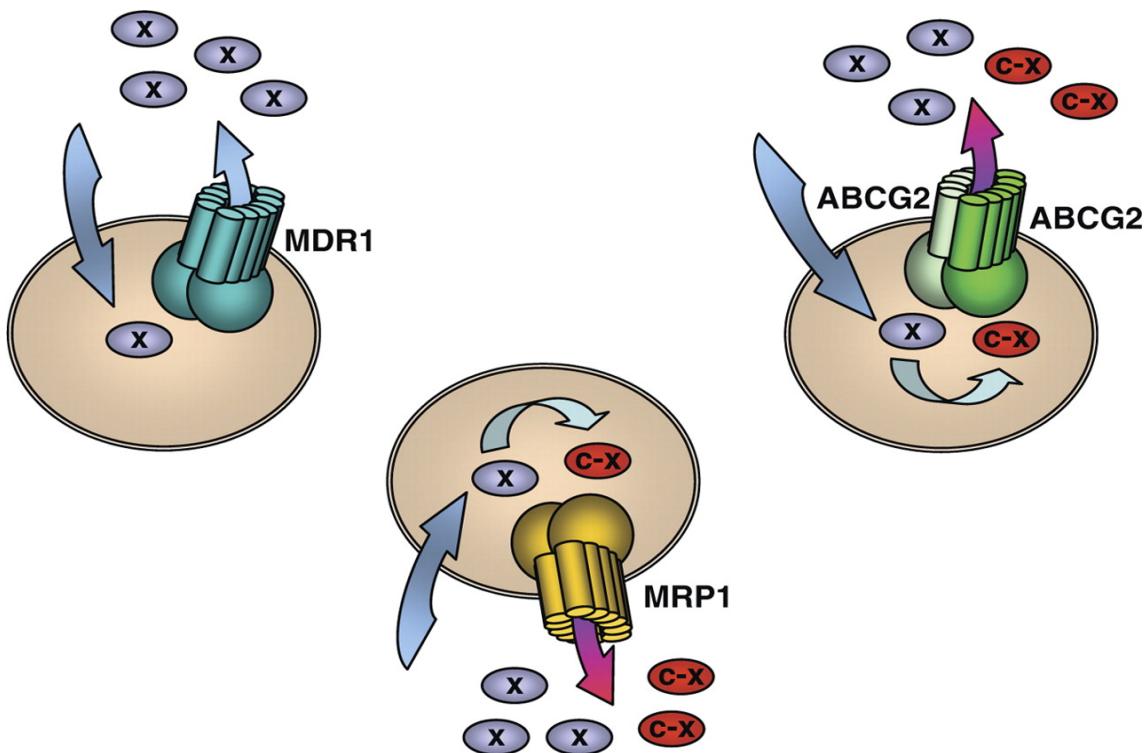


Figure 1.1. Function of the multidrug/xenobiotic ABC transporters. Multidrug/xenobiotic ABC transporters reside in the plasma membrane and extrude various hydrophobic and/or amphipathic xenobiotics and metabolic products. MDR1/Pgp transports hydrophobic compounds (X), while MRP1 and ABCG2 can extrude both hydrophobic drugs and intracellularly formed metabolites, e.g., glutathione or glucuronide conjugates (C-X).

P-gp and MRP transporters are already described as active in several aquatic organisms including marine mussels (Luckenbach and Epel, 2005; Luedeking and Koehler, 2004), freshwater mussels (Faria *et al.*, 2011), sea urchins (Kurelec, 1992) and fish (Bard, 2000). Transcriptomic analyses of those transporters show high homology between them and humans (Sturm *et al.*, 2009).

In environmental sciences the majority of the research efforts have been focused in xenobiotic transformation and elimination. These transporters constitute the first and last line of defence against contaminants. In the first line are ABCB (P-gp transporters) and some ABCC (MRP ones) that effluxed out chemicals when enter to the cell. MRP transporters are also known to effluxed out xenobiotic metabolites and conjugates (Epel *et al.*, 2008), acting as a last line of defence.

1.4.1. ABCB family (P-Glycoprotein or p-gp)

The P-gp is the best studied eukaryotic ABC transporter. It is responsible for multidrug resistance (MDR) phenomenon involved in tumour cells resistance to most chemotherapeutic drugs.

In aquatic organisms, the first report of P-gp (MDR1 like) mediated toxicant resistance was in the freshwater mussel *Anodonta cygnea* (Kurelec and Pivčević, 1989). Since then it has been identified in up to 35 aquatic organisms such as, marine and freshwater mussels, clams, crabs, fishes, worms, sea urchins among others (Bard, 2000; Faria *et al.*, 2011; Smital *et al.*, 2003). P-gp is a 170kda size transporter protein and recognizes an extraordinary wide spectrum of chemicals which is also characteristic of other MXR transporters; the subfamily contains both full and half transporters (Dean and Allikmets, 2001; Sturm *et al.*, 2009). P-gp is remarkably non-specific with respect to its substrates. This lack of specificity is of high adaptive importance and generates protection against many novel anthropogenic products.

Contrary to other ABC transporters, P-gp does not efflux metabolites, but the parent compound alone is the transporter substrate. P-gp localization within the membrane suggests its action on the substrate shortly after it enters the cytoplasm. P-gp has high affinity to moderately hydrophobic, amphipathic (somewhat soluble in both water and lipids) chemicals of low molecular weight, cationic or neutral, planar with basic nitrogen atoms, and natural products (Endicott and Ling, 1989; Gottesman and Pastan, 1988; Gottesman *et al.*, 1994). In humans P-gp proteins and RNA are detected in high levels in liver, kidney, small bowel, colon, pancreas, adrenal cortex, placenta, blood brain barrier tissues (Cordon-Cardo *et al.*, 1990; Fojo *et al.*, 1987; Sugawara, 1990; Sugawara *et al.*, 1988). In aquatic invertebrates it has been identified in tissues involved in absorption, secretion and barrier function, such as gills, anterior digestive tract and epidermal tissue of worms, cell membranes of sponges and others (Bard, 2000).

1.4.2. ABCC family (Multiresistance protein or MRP)

Multiresistance proteins (MRPs) belong to the C family of ABC transporters. ABCC subfamily consists of a diverse group of 190kDa glycoproteins. In this

family there is also full and half transporters with 3 TMDs (Bard, 2000; Sturm *et al.*, 2009). The MRP have a very broad substrate specificity which includes many organic anion conjugates of structurally different compounds such as endogenous substrates that are natural products of the metabolism and xenobiotic metabolites resulting from phase I and II of the biotransformation metabolism. The transport of several of these conjugates has been shown to depend on the presence of reduced Glutathione (GSH) (Cole and Deeley, 2006). Other functional properties also include iron transport, cell surface receptors and toxin secretion activities. Additionally, some members of the MRP family also contribute to the efflux of unmodified hydrophobic compounds, thus acting in a similar way as P-gp.

1.4.3. ABCG family (Breast Cancer Resistance protein or BCRP)

ABCG is composed of several members, where ABCG2 is the most prominent and better characterized. BCRP/ABCG2 confers resistance to a narrower range of substances than P-gp or MRP. ABCG is a 72kDa “half transporter” protein, consisting of only one NBD and one TMD (Sturm *et al.*, 2009; Wakabayashi *et al.*, 2006). To achieve full function, two of these half transporters have to dimerize in order to form a fully active transporter (Ewart and Howells, 1998). Like P-gp, BCRP is not dependent on GSH complexation for recognition and transport of its substrates. ABCG2 is prominently expressed in apical membranes of polarized epithelial cells of different organs involved in absorption (small intestine), distribution (placenta and blood brain barrier) and elimination (liver)(Ito *et al.*, 2005), indicating its important role in excretion of xenobiotics. It is also implicated in the transport of important physiological molecules like cholesterol, and has been shown playing an important role in the metabolism of *haeme* groups (Doyle and Ross, 2003; Latunde-Dada *et al.*, 2006).

1.4.4. Chemosensitization related to MXR

A large number of anthropogenic pollutants such as PAHs, PCBs, DDT, musks and their metabolites can either induce or inhibit the transporter activity

of these proteins in fish, invertebrates and mammals (Luedeking and Koehler, 2004; Minier *et al.*, 1993). Chemicals able to inhibit MXR/MDR transporter activities are termed chemosensitizers (Cornwall *et al.*, 1995; Smital *et al.*, 2003) and the phenomenon is called chemosensitization. A consequence of chemosensitization is the increase accumulation of xenobiotics and metabolites inside the cell, and therefore enhanced toxic effects. Chemosensitizers can be chemicals that are not necessarily toxic themselves, but are also substrates of the transport system and thus compete with other more toxic xenobiotics saturating the transport system and compromising the MXR defence(Kurelec *et al.*, 2000). Chemosensitizers tend to be more lipophilic than other substrates (Sharom, 1997). Xenobiotics that are substrates of the MXR transporters are found experimentally to diffuse very slowly across membranes on the order of minutes to hours when compared to chemosensitizers, which transverse membranes much faster (Eytan and Kuchel, 1999). Other chemosensitizers may not be substrates to the transporters pumps, but nevertheless inhibit their activity through some indirect effects (i.e. inhibiting ATP) (Epel *et al.*, 2008).

Chemicals that can interact with these efflux transporters may not only modulate the toxic potential of the xenobiotics but may also interfere with important biological processes. (Luckenbach *et al.*, 2004) have shown that synthetic musk's have the effect of blocking these transporters in mussels. These musks are used as fragrances in many personal care products and end in the wastewaters, thus they may pose a serious environmental threat, by changing how aquatic organisms are able to deal with other xenobiotics and even their own metabolites.

Chemosensitization mechanisms can be of extreme importance on how organism deal with toxicity and any substance that can impair this system can be of extreme importance. Often, the chemosensitizer agent is not toxic itself, but in a mixture with other toxic compounds, will increasing their accumulation within cells and hence enhancing their toxicity. These factors are even more important in aquatic organisms and can only be addressed if we change our common assessment of the impact of any given chemical individually, by adding also mixture testing methodologies.

1.4.5. Chemosensitization and Mixture toxicity

In natural environments, organisms are frequently exposed to mixtures of pollutants (Sharom, 1997) and are relatively uncommon to find sites polluted with only one substance (Walker, 2001). Some chemicals are highly persistent, some are applied repeatedly or continuously, and others are applied as mixtures to increase efficiency or reduce costs (Marking, 1977). Due to the large number of different chemical compounds present in the environmental matrices, the individual testing of each component is not fully representative of the total mixture effect. Thus, robust predictive toxicity models are needed, in order to estimate their toxicity with acceptable accuracy (Lydy and Austin, 2004; Vighi *et al.*, 2003).

In order to address the effect of a toxic mixture, the observed combined toxic effect can be compared with an expected combined effect calculated from the single component toxicity, using specific reference models. Specifically in environmental toxicology, there are two well established conceptual non-interactive models, termed Independent Action or response addition (IA) and Concentration Addition (CA), derived from pharmacological sciences. These two models describe general relations between the effects of single substances and their corresponding mixtures, for dissimilar and similar acting chemicals (Barata *et al.*, 2007).

The Independent action (IA) model is a statement about the relationships between the probability of the response, whereas the concentration addition (CA) model refers to relative toxicities (De March, 1987). In other words, independent action addresses the question whether the probability of response to one chemical may be independent from the probability of response of another, while concentration addition addresses the question if the relative toxicity of the mixture is the same as the sum of the toxicities of the individual compounds. In aquatic toxicology the IA and CA have been successfully used to predict non interacting joint effects of mixtures of dissimilar and similar acting compounds (Altenburger *et al.*, 2000; Backhaus *et al.*, 2000; Barata *et al.*, 2007; Faust *et al.*, 2003; Faust *et al.*, 2000; Scholze *et al.*, 2001).

To predict accurately joint effects of mixtures it is crucial to estimate accurately the predicted values for individual and mixture combinations (Scholze *et al.*, 2001). Predicted values for the studied individual components

can be estimated using several approaches and models. One of them is to use the best fit model (Scholze *et al.*, 2001). One of the most used models is the non-linear Hill equation (eq1), which according to the rate theory(Eyring, 1935) can be used to describe the inhibition of a biological process as a function of toxicant concentration using a sigmoid type function. When considering mortality values that range between 0 and 100 the Hill model become that of Eq 1.1.

$$E(\%)_{inh} = \frac{100}{1 + (EC_{50}/x)^p} \quad eq. \quad 1.1$$

with E=effect in %; p=slope; EC=effect concentration; x=concentration (μM).

On the basis of the concentration-response functions of individual compounds, predictions of concentration addition were calculated for mixture containing binary combinations in a definite ratio (based on EC50 or EC10). A total concentration of the mixture, at which a certain effect is generated, can be calculated using concentration addition according to Eq 1.2:

$$ECx_{mix} = \left(\sum_{i=1}^n \frac{p_i}{ECx_i} \right)^{-1} \quad eq. \quad 1.2$$

In this equation ECx_{mix} is the total concentration of the mixture provoking $X\%$ effect; ECx_i is the concentration of component i provoking the $x\%$ effect, when applied singly; and p_i denotes the fraction of component i in the mixture. The calculation of total mixture concentrations for various effect levels lead to a complete iteration of an expected concentration–effect relationship.

The prediction concept IA allows explicit calculation of combined effects according to Eq 3:

$$E(c_{mix}) = 1 - \prod_{i=1}^n (1 - E(c_i)) \quad eq. \quad 1.3$$

The effect at the total concentration of the mixture, $E(c_{mix})$, is based on the effects of the components which they generate at concentration x at which they are present in the mixture ($E(c_i)$). If the latter is expressed as a fraction (p_i) of the total mixture concentration, it holds Eq 4:

$$E(c_{mix}) = 1 - \prod_{i=1}^n (1 - E(p_i c_{mix})) \quad eq. \quad 1.4$$

This allows calculation of an effect expected according to the concept of IA for any concentration of the mixture.

These models can be of key importance when trying to characterize the toxicological role of MXR system, allowing to understand if the inhibitors, by blocking the capacity of transport, increases the toxicity of the substrates (Faria *et al.*, 2011).

1.5. Ecotoxicology and genomic tools.

The biological impact of pollutants on ecosystems is the result of a cascade of events that begins with their introduction into the environment. Environmental pollution affects different biological processes within the exposed organisms causing adverse effects in target tissues, whole organism and eventually in entire ecosystems (Piña *et al.*, 2007). When toxic pollutants enter an organism they trigger physiological responses aimed to maintain homeostasis. The first response is at gene level and then transferred to higher organizational levels until a final, ecosystem level (Van der Oost *et al.*, 2003).

A major mechanism underlying almost all the responses to environmental change is mediated by a shift in the transcription of genes.

Genes, the building blocks of life, are regions of the DNA chain, that contain the blueprint for proteins. According to the fundamental dogma of biology, genes are transcribed to mRNA (messenger RNA), and this is then translated into proteins. Proteins are the elements that determine structure and functionality of all living organisms, therefore, every response to change, chemical or not, always requires a change of the gene transcription.

A major step for our ability to study and understand, the transcriptional patterns of genes came with the development of PCR (Polymerase Chain Reaction), developed by Kary Mullis and Michael Smith in 1983 (Bartlett and Stirling, 2003). This technique revolutionized the way how we were able to study genomic processes. With PCR, and more recently with quantitative Real-Time PCR (qPCR), we became able to study many new potential genetic biomarkers, being that every gene is a potential biomarker of any given change: life-cycle changes, chemical changes, etc.

In 2004 the term “*Ecotoxicogenomics*” was introduced and defined as the integration of genomic based science into ecotoxicology studies (Snape *et al.*,

2004). The first obvious advantage of using genomic information was the increase of the information that we could extract from an experiment, thus allowing for better and more accurate conclusions. This would also allow us to link molecular biomarkers with higher level population and ecosystem responses, and that way anticipate potential risks. This new branch is now developing into a key tool for the assessment of environmental impacts and Environmental Risk Assessment, because of its potential to highlight toxicant specific gene expression patterns that can be used to identify major change inducing chemicals. Genomic technologies are also having a decisive contribution to the development of new biomarkers, and the determination of new and more accurate Mode-of-Action (Piña and Barata, 2011).

Microarrays are small, solid supports hosting thousands of single stranded DNA sequences called probes, in which each probe represents a gene of interest. Supports are normally glass slides, silicon chips or nylon membranes, and probes are printed or attached in a known and fixed location. Hybridization between these probes and complementary target sequences labelled with a fluorescent dye is the principle behind microarrays technique.

Conditions in which hybridization and subsequent washings are carried out, promote the formation of only strongly paired strands, that is, those with a high number of complementary base pairs, avoiding non-specific interactions. Total fluorescent signal emitted by each spot depends upon the amount of target sequence binding to the probes present on that spot. Scanning of the microarray and subsequent analysis of the resulting image allows the quantification of the intensity values from each spot, which are proportional to the initial amount of target sequence in the sample.

Microarray-based gene expression analysis can be used to identify multiple genes whose expression change due to a particular influence factor. Target sequences are obtained by reverse transcription and labelling of mRNA isolated from the samples that will be compared. Fluorescence intensity values obtained by the analysis of images represent the expression levels of genes in a given condition. Comparison of these gene expression patterns allows the identification of genes whose expression change due to the factor under study. Although microarray technology has a very high throughput interrogating

thousands of genes simultaneously, the process includes numerous sources of variability being necessary to apply statistical tools in experimental design and in data analysis. This is essential to obtain high quality results from microarray experiments (Draghici *et al.*, 2003). Final confirmation of the interest genes quantitative change may be done using qPCR, for a more robust analysis.

A full genome gene analysis can be made with only one chip, giving the researchers a very comprehensive dataset. One of the most appealing characteristic of the microarrays technique is that it's main, and probably single limitation is our knowledge of the analysed species (Piña and Barata, 2011).

The final step of the microarray experiments is linking the gene effects to biological functions. With the development of bioinformatics tools like Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEEG), we are now able to better understand how the exposure scenario changes the gene transcriptional patterns and link these to metabolic and regulatory pathways. Genes modify their expression as to maintain cell homeostasis in a compatible way with the external impact, and this process implies that genes sharing a common pathway or under the same control mechanism, would change their transcription in a coordinated manner (Piña and Barata, 2011).

The GO (www.geneontology.org) principle, is that genes with the same biological and/or molecular functions are grouped together through a controlled vocabulary of terms, which can be used across species taking into account the gene product attributes. By analysing the Differentially Expressed Genes (DEG) in this way, we are able to have a better understanding of the potential pathways and functions affected, thus, giving a much robust sight of the MoA. The KEEG (<http://www.genome.jp/kegg/>) analysis works in a similar way, with the advantage of allowing us to position our genes in molecular pathways and understand the potential up and downstream effects.

Genomic tools results evidence important achievements such as an increased ability to characterize toxicant specific genes expression patterns, selecting new molecular biomarkers and in many cases in determining putative mechanisms of action (Piña and Barata, 2011).

1.6. Biomonitoring of aquatic organisms

Assessing the ecological risks of emerging pollutants requires an understanding of both the exposure regime and the effects of the chemical on different organisms (Fent *et al.*, 2006). For example, some ECs can act as endocrine disrupting chemicals (EDCs), with the potential to affect hormonal driven processes, such as reproduction and/or development. Aquatic organisms are particularly susceptible to EDCs, because their entire life cycles are spent in continuous contact with water. Despite that ED mechanisms are well known in vertebrate species, little is known on invertebrates (Barata *et al.*, 2004). According to the previous authors, reported inhibitory effects on reproduction and/or growth were likely to be related to toxic effects on energy supply or demand, rather than to a disruption of the endocrine system itself. However, several emerging pollutants may affect aquatic invertebrates in unexpected ways, more similar to the canonical endocrine disruption as defined in vertebrates, like increasing male production or the reproductive output (Flaherty and Dodson, 2005; LeBlanc *et al.*, 2000b; Olmstead and LeBlanc, 2000). In relation to this, there is an urgent need to develop sensitive bioassays to measure unambiguously specific effects from emerging contaminants (Barata *et al.*, 2004) and then, to study the underlying mechanisms.

There is an increase degradation of the water bodies worldwide, due to the release of chemicals, including the ones of emerging concern, which may be posing new threats on the ecosystems, affecting their health and balance. In this context elucidating potential adverse effects of complex mixtures of chemicals, together with confounding factors constitutes a new challenge for the scientific community.

The inherent difficulties of field studies, where potential interactions among chemicals might occur, together with the scarce information available on the development, physiology, biochemistry and genomics of most non-model aquatic organisms, make the identification of sublethal toxic effects extremely challenging. Mitigation of these problems requires an increased knowledge about the physiology of the test animals.

1.7. *Daphnia magna* as a test organism

For the execution of this thesis, we use *Daphnia magna* as model organism.

Daphnia is one of the most used organisms in environmental research. A simple search in the WEB OF KNOWLEDGE retrieves more than 12000 papers published from the year 2000 to present, with reference to *Daphnia* of which, more than half are related to *D. magna*. The taxonomy classification of *Daphnia magna* Strauss, accordingly to the World Register Of Marine Species (Boxshall, 2013) is as follows:

- Kingdom: Animalia
- Phylum: Arthropoda
- Class: Branchiopoda
- Subclass: Phyllopoda
- Order: Diplostraca
- Suborder: Cladocera
- Infraorder: Anomopoda
- Family: Daphniidae
- Genus: *Daphnia* O.F. Muller, 1785
- Species: *Daphnia Magna* Straus, 1820

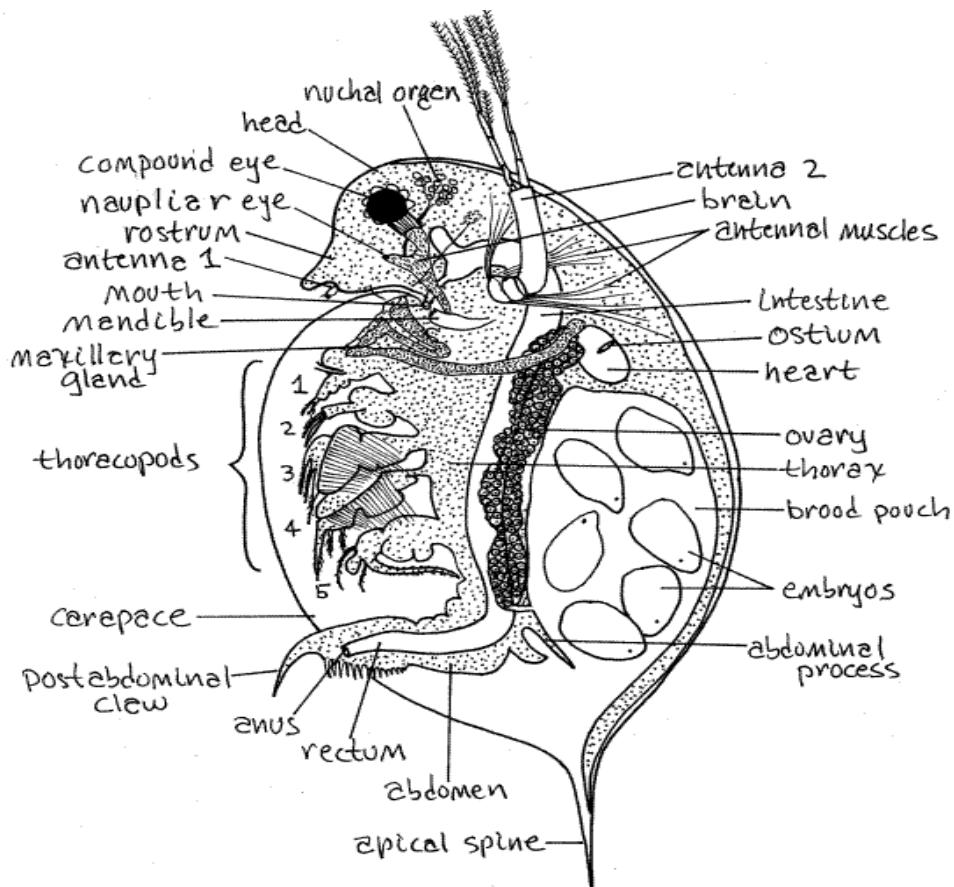


Figure 1.2. The functional anatomy of *Daphnia* (From Richard Fox, Lander University)

D. magna is a brackish and freshwater organism. Anatomy and morphology are shown in Fig 1.2. Like all crustaceans, it possesses an exoskeleton, consisting of a dimerous chitinous carapax, with multiple tissue layers, surrounding the thorax and abdomen. Like most crustaceans, the carapax is non-elastic, as a consequence of which growth is possible only by regular renewals of it, a phenomenon known as molting (Ebert and Jacobs, 1991). *D. magna* has two pairs of antennas: the first is known to function as sensory organ and the second are modified for swimming. Due to the position and movement of the second antenna, *Daphnia* is mainly restricted to vertical movements, presenting its characteristic movement of hop-and-sink.

In the head region, there are the composed eye, the brain, connected to the nauplius eye and the gut opening. Directly in front of the gut opening, there is a pair of maxillae, rigid, which are able to triturate, to a certain degree, the food particles. The gut has an upward orientation in this section, making a U in its

higher point, and then directing towards the posterior section of the body. In the head region, connected to the gut, there is a pair of caeca, which are homologous to a hepato-pancreas (Ebert and Jacobs, 1991).

In the thoracic region are present the filter setae, used as filters, retaining the food particles and directing them to the maxillary glands, epipodites (long regarded as gills, but actually are sites of haemoglobin production;(Goldmann *et al.*, 1999)), heart and 2 ovaries, flanking the gut; and the brood pouch, an opening on the back of the animals where the embryos will be deposit and develop till birth. The post-abdomen is bent ventrally (as the entire animal) and forward, finishing in a spine. It possesses a claw and spines, which uses to clean the thoracic appendages and carapax, and to keep the eggs in the brood pouch (Ebert and Jacobs, 1991).

D. magna has a Holarctic distribution, living in small or medium size shallow freshwater ponds with low fish predation pressure (De Gelas and De Meester, 2005). It represents a large part of the zoo-planktonic community, being a keystone species in the freshwater ecosystems. It's one of the most important primary consumers, being fundamental to control algae blooms, while is also important as a food source for both invertebrates and vertebrates predators. The presence of *Daphnia* has considerable implications for the maintenance of the ecological quality status of aquatic systems (Hebert, 1978). *Daphnia* species are non-selective filter feeders, using their thoracic appendages to create water current through the interior of the carapax using their fine *setae* to retain particles, which are then transported to the *maxillae*. *Daphnia* sp. mainly feed on algae, but will retain all the suspended particles that can be withheld by their filtering apparatus (>1um), Ingesting any suspended particles (Gillis *et al.* 2005).

D. magna reproduces through cyclical parthenogenesis (Zaffagnini, 1987). During the parthenogenic phase, the population is composed exclusively by females, which produce diploid eggs in the ovary (Figure 1.3). Oogenesis is not fully meiotic nor strictly mitotic resolving in diploid eggs identical to the mother. The eggs are then deposited in the brood pouch, in which they will develop to clones that are genetically identical to the mother. They maturation is complete in around 3 days (at 20°C) and are released to the environment by contraction

of the post abdominal part of the body (Ebert, 1994). Neonates are similar to the adult, with around 0.6-1 mm in length (top of the head to start of the abdominal spine). The neonates will then undergo 4-5 molting stages, and will be sexually mature at 6-8 days at 20° and optimal food conditions (Ebert, 1994).

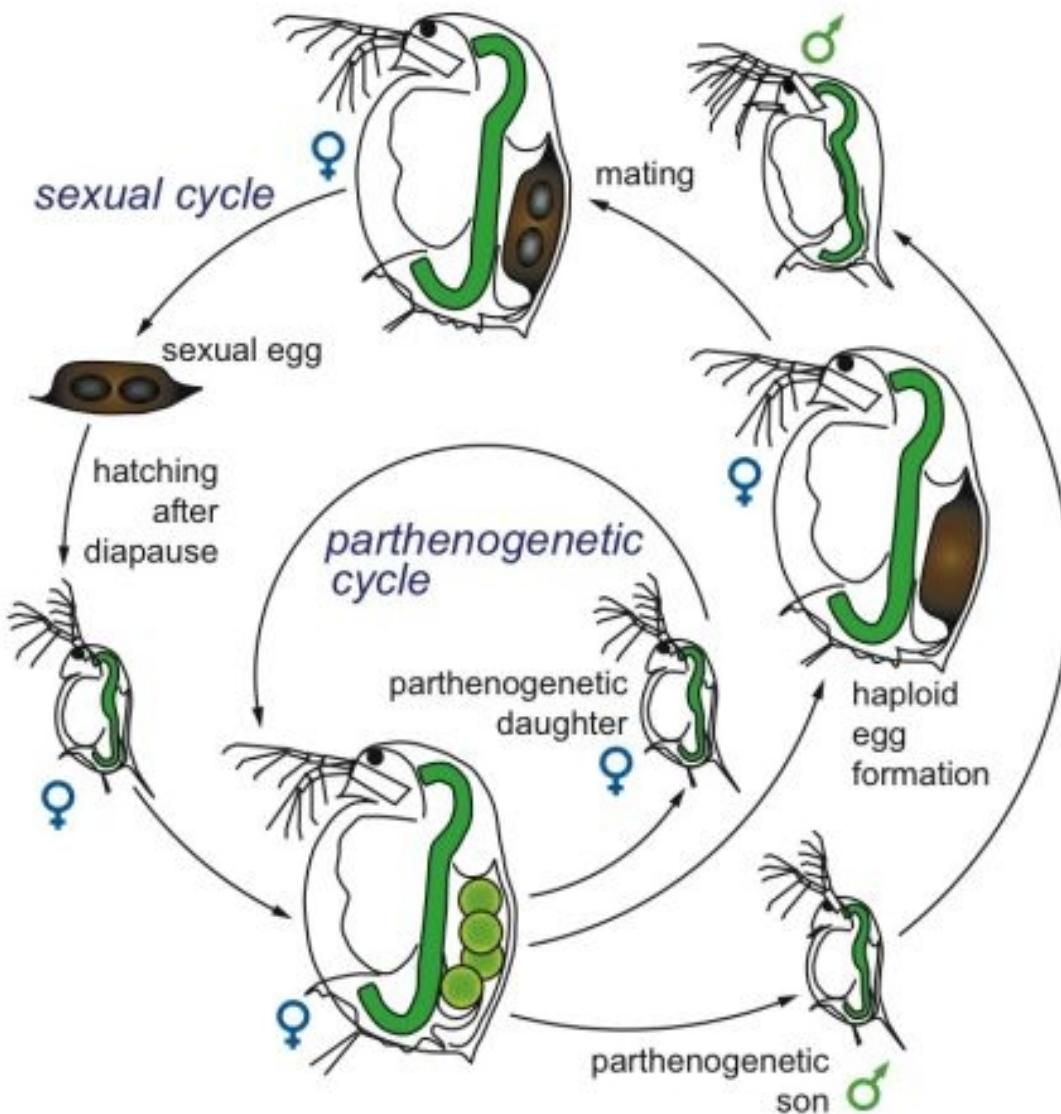


Figure 1.3. Life cycle of a *Daphnia* (from Ebert 1995)

Even though *Daphnia* mainly reproduces through parthenogenesis, certain environmental triggers like food limitation, high population densities, short photoperiod or desiccation of the habitat can activate sexual reproduction. The males are formed during a special parthenogenetic event, and therefore they are genetically identical to the mothers (Figure 1.3). They are identical to the females in aspect, but are of smaller size, and have the first antenna much more developed. Subsequently, females produce haploid eggs (2 per brood)

that are deposited in the brood pouch, which are then fertilized by male haploid spermatozoa and then undergo a further development to resting eggs, also called ephipia (figure 1.3). These resting eggs are encapsulated by multiple layers, formed by a transformation of the brood pouch in order to protect the eggs inside against adverse environmental conditions. They are able to resist in dormant status for up to hundred years (Jeppesen *et al.*, 2001). When the conditions become favourable, the ephipia develop to parthenogenically reproducing females, and a new population is established.

In laboratory, optimal conditions for the *Daphnia* development can be maintained continuously, allowing the elimination of sexual reproduction events and creating the possibility of keeping clones indefinitely. This give to researchers a very important tool that is the possibility of studying an organism that is a perfect genetic clone, with all its implications at the level of reproducibility of experiments and constancy of results between tests and a good model for studying genomic variations without the problem of normal variation in species with sexual reproduction events.

D. magna has been studied for almost three centuries in the fields of taxonomy, physiology and limnology, which has provided useful information for all the modern research. *Daphnia* can be found in freshwater ecosystems all around the world, playing a key ecological role in the food web, resulting in the recognition of the ecological relevance of testing. The lifecycle is ideally suited for experiments, due to its short reproduction time, when compared to most eukaryotic model species: the reproductive maturity is reached within six to ten days and a reproductive batch occur every three days hereafter (Ebert, 1992). Through asexual parthenogenic reproduction, it is possible to produce genetically constant clonal lines, hereby reducing genetic variability, which can be a factor of interest in many experiments. On the other hand, different clones can be maintained to create experimental populations with controlled genetic variation.

D. magna is the most frequently used invertebrate in standard acute and chronic aquatic toxicity testing. Bioassays with *D. magna* are formally endorsed by major international institutions such as the European Union, EPA, the OECD

and ISO. Results from these assays have been used in environmental risk assessment screenings of almost all substances.

1.7.1. Genomic information and molecular tools available

The genome of the related species *D. pulex* has been sequenced recently by the joint efforts of several research groups integrating the *Daphnia* Genomics Consortium (Colbourne *et al.*, 2011). The *D. pulex* genome has more than 31000 genes detected, with an estimation of 8000 not detected. For *D. magna* there is an on-going effort for sequencing the whole genome and this is planned to be accomplished in short time. Nevertheless, there is extensive data on genomics of *D. magna*, with more than 15000 EST (Expressed sequence tags) and more than 1600 protein sequences.

D. magna microarrays have been developed since 2004 by different groups. The first microarray was developed in the University of Antwerp. It was a cDNA microarray, based on energy, molting and reproduction related cDNA libraries obtained by SSH (suppressive subtractive hybridization)(Soetaert *et al.*, 2006; Soetaert *et al.*, 2007a). This technique allows for the selective amplification of differentially expressed transcripts, e.g., between neonates and adult reproducing population (reproduction), while suppressing the amplification of the transcripts that are present in both populations. The same method was followed for the other two libraries. These microarrays were subsequently successfully used in ecotoxicogenomics studies with *D. magna* (De Schampelaere *et al.*, 2008; Dom *et al.*, 2012; Soetaert *et al.*, 2007a; Soetaert *et al.*, 2007b; Vandebrouck *et al.*, 2009). Another group has developed an Oligonucleotide microarray, based on ESTs (expressed sequence tags)(Watanabe *et al.*, 2007). This database was developed during studies in which double stranded cDNA was derived from RNA of three to four weeks old daphnids. These cDNA fragments were then cloned and sequenced, and obtained 7210 EST of which almost 3000 were non redundant. Of these fragments 41% displayed homology to genes deposited in the NCBI database (Watanabe *et al.*, 2008). This set of EST was then combined with the previous Belgium libraries and enriched with cDNA obtained with SSH from populations exposed to different toxicants, pH and calcium limitations to build a larger microarray (Connon *et al.*, 2008). This version of the microarray was then used

in a systems biology approach to detect transcriptomic and phenotypic stress responses, in order to unravel the MoA (mode of Action) of *D. magna* exposed to ibuprofen (Heckmann *et al.*, 2006; Heckmann *et al.*, 2008a). Another step forward was made by a group in the United States, which developed a custom cDNA microarray, based on a random selection of 5000 cDNA clones from the *Daphnia* Genomics Consortium (Poynton *et al.*, 2008), enriched with 2681 cDNA transcripts, obtained by SSH from daphnids exposed to munitions constituents and metals with success in many studies after (Garcia-Reyero *et al.*, 2012; Poynton *et al.*, 2012).

1.8. Objectives:

Environmental Risk Assessment (ERA) of emerging pollutants requires the development of bioassays able to detect novel mechanisms of action of Emerging Contaminants. This approach puts a new focus on the study of sublethal effects with an ever increasing sensitivity of the assays used.

During the development of this thesis, I will focus on detecting and understanding sublethal effects and propose to develop and use novel bioassays to unravel the Mode of Action (MoA) of several Emerging Contaminants, namely pharmaceuticals, 4-nonylphenol and nanoparticles in *Daphnia magna*.

For all the studies planned, I aim to use standard tests, coupled with new custom designed ones, in order to achieve the sensitivity levels needed to detect the potential sublethal effects.

I divide this thesis in three main objectives:

Objective one:

Study the effects of two antidepressants (Selective Serotonin Reuptake Inhibitors) and 4-nonylphenol in *Daphnia magna*. Linking population, physiological and genomic studies to unravel Modes of Action.

Chapters 2, 3 and 4 present data obtained from several experiments executed to assess responses at the individual and population level, but also at the physiological and genomic levels, of animals exposed to the SSRIs (fluoxetine and fluvoxamine) and 4-nonylphenol. The proposed experimental framework allowed determining the Mode of action (MoA) of these substances across biological levels of organization using *D. magna* as a model organism.

Objective 2:

Study the effects of Titanium dioxide Engineered Nano Particles on food depletion and life history responses of *Daphnia magna*.

In chapter five, I attempt to discriminate the toxic effects of NM to *D. magna*, by distinguishing direct toxicity from indirect effects due to their aggregation with edible particles, depleting food levels and hence causing starvation of exposed individuals. I also propose to develop new assays that can be used to characterize other NM and its aggregation mediated effects to other water column grazers such as many zoo-planktonic species.

Objective 3:

Characterize the Multixenobiotic Resistance system (MXR) in *Daphnia magna* and its toxicological role”

In chapter six I propose to characterize the MXR system in *D. magna* for the first time. The study was performed at the gene level sequencing partial sequences of the more important genes and studying their transcriptional patterns across developmental stages and inducers. The development of transporter assays using fluorescence dyes allowed to assess the functionality of the new sequenced genes. Finally the use of binary mixture toxicity assays combined toxic substrates of the different MXR system with specific inhibitors of that pumps help me to study the toxicological role of the MXR system in *D. magna*.

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CHAPTER 2

**Enhanced offspring production in *Daphnia magna*
clones exposed to serotonin reuptake inhibitors and 4-
nonylphenol. Stage- and food-dependent effects**

Enhanced offspring production in *Daphnia magna* clones exposed to serotonin reuptake inhibitors and 4-nonylphenol. Stage- and food-dependent effects

Bruno Campos, Benjamín Piña, María Fernández-Sanjuán, Silvia Lacorte, Carlos Barata

Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona, 18-24, 08034 Barcelona, Spain

Published: Aquatic Toxicology 109 (2012) 100-110

Abstract

Risk assessment of emerging pollutants requires the development of bioassays able to detect and understand novel mechanisms of action. This study tested the hypothesis that the increase of offspring production in *Daphnia magna* induced by certain pollutants may be mediated through different mechanisms, depending on development stages, clones and food rations. The study included two selective serotonin reuptake inhibitors (SSRIs), fluoxetine and fluvoxamine, and the detergent metabolite 4-nonylphenol. Organisms were exposed from birth to adulthood or only during adulthood at low and high food ration levels. Results indicated that low exposure levels of the three studied substances increased offspring production and/or juvenile developmental rates similarly for all studied clones, but the responses differed among life-stages and food rations. When individuals were exposed to the studied chemicals from birth, enhanced offspring production per female was observed only at low and intermediate food rations. On the contrary, when exposures started in gravid females most treatments increased offspring production. Results obtained with SSRIs support previous findings, where it was stated that these compounds may amplify serotonergic signalling in *D. magna*. 4-Nonylphenol effects may be related to the reported alteration of this compound in *Daphnia* ecdysteroid metabolism. Further investigations are necessary to resolve the biochemical mechanism of SSRIs and 4-nonylphenol enhancing offspring production.

Keywords: Pharmaceuticals, 4-Nonylphenol, Reproduction, Growth, Selective Serotonin Reuptake Inhibitors (SSRIs), *Daphnia*

2.1. Introduction

Assessing the risks of emerging pollutants requires an understanding of both the exposure regime and the effects of the chemical on different organisms (Fent *et al.*, 2006). Of special concern are those emerging pollutants that may act as putative endocrine disrupters in invertebrate species (Barata *et al.*, 2004). Most known vertebrate endocrine disruptors act in a dose or concentration response fashion in aquatic invertebrates by impairing survival, growth or/and reproduction (Barata *et al.*, 2004). According to the previous review, reported inhibitory effects on reproduction and/or growth were likely to be related to toxic effects on energy supply or demand, rather than to a disruption of the endocrine system itself. However, several emerging pollutants may affect aquatic invertebrates in unexpected ways, more similar to the canonical endocrine disruption as defined in vertebrates, like increasing male production or the reproductive output (Flaherty and Dodson, 2005; LeBlanc *et al.*, 2000a; Olmstead and LeBlanc, 2003). The latter is usually referred as a hormesis effect in ecotoxicological evaluations (Calabrese, 2008) and should be carefully analysed in the context of endocrine disruption (Barata *et al.*, 2004) and life-history evolution (Forbes, 2000). In relation to this, there is an urgent need to develop sensitive bioassays to measure unambiguously specific effects from emerging contaminants (Barata *et al.*, 2004) and, then, to study the underlying mechanisms. *Daphnia magna* is an excellent model species to study unknown mechanisms of action of emerging pollutants. *D. magna* is widely used in aquatic toxicology and ecology, thus its expected response to a broad diversity of contaminants and environmental factors is well known. Several recent studies have characterized with great detail specific effects of pollutants that disrupted juvenile and moulting endocrine systems in *D. magna*. These studies have shown that endocrine disruptors act differently on eggs, juveniles and adults, disrupting egg development, juvenile growth and the sex of offspring (Leblanc and McLachlan, 1999; LeBlanc *et al.*, 2000b; Mu and LeBlanc, 2002; Olmstead and Leblanc, 2002, 2003). On the other hand, information on pollutants able to disrupt reproduction in an unexpected way, enhancing reproduction rather than inhibit it, are scarce and contradictory. Enhanced offspring production in *Daphnia* females exposed to pollutants has been

reported for fluoxetine, 4-nonylphenol and dispersogen A (Brooks *et al.*, 2003a; Brooks *et al.*, 2003b; Hammers-Wirtz and Ratte, 2000; LeBlanc *et al.*, 2000b), although inhibitory effects on reproduction have also been found for the first two chemicals (Baldwin *et al.*, 1997; Hansen *et al.*, 2008). These contradictory results may indicate that these chemicals may act differently across developmental stages, clones and/or exposure levels. For example, it could be hypothesized that they target key processes which control growth and reproduction and, consequently, they may act differently during pre-adult and adult stages, when energy allocation to growth and reproduction are prioritized, respectively (Nogueira *et al.*, 2004). Alternatively, as pointed out by Brooks *et al.*, 2003b, stage-specific toxic effects of fluoxetine could be associated with an age-dependent developmental increase in its P-450 metabolism, to produce the active metabolite, norfluoxetine. Similarly, clones having different growth and reproductive performance under laboratory conditions may also be affected differently by pollutants targeting those processes (Baird *et al.*, 1990). Food quantity also affects growth and reproduction of *D. magna* (Barata and Baird, 1998). As the above mentioned chemicals and food affect the same life-history traits, we might expect interactions among them. According to the life-history theory (Sibly and Calow, 1986), organisms optimize their key physiological functions: maintenance, growth and reproduction, to maximize their fitness under a limited food resource environment. As a result, life-history responses are constrained within narrow limits due to the existence of trade-offs. Important ones are reproduction versus growth and offspring size and number. This means that in an environment with given food resources, increasing investment in reproduction is counteracted by reduced growth and mothers may produce more but smaller offspring. Additionally, *D. magna* has the ability to vary the number of developmental instars (here-after referred as maturation instars) invested until maturity and hence the timing, body and clutch size at first reproduction (Barata and Baird, 1998; Ebert, 1994). In conclusion, toxic chemicals may enhance offspring production at expenses of growth and offspring size. There are few studies reporting that enhanced offspring production in *D. magna* was related with smaller offspring or decreased fecundity with increasing growth (Hammers-Wirtz and Ratte, 2000; Heckmann *et al.*, 2007). This study aims to test the hypothesis that effects of contaminants

with reported evidence for increasing offspring production in *D. magna* are stage-dependent (juvenile vs. adults) and that the effects vary across clones and food rations (from low to high). Selected pollutants included two selective serotonin reuptake inhibitors (SSRIs), fluoxetine and fluvoxamine. Despite their different chemical structure, both chemicals have the same common mode of action by blocking the re-uptake of serotonin in the nerve synapses. This effect is used worldwide to treat clinical depression in humans (Rang *et al.*, 1995), and, consequently, these compounds are nowadays widespread in the environment. For example, fluoxetine and other SSRIs have been detected broadly in water and tissues of organisms (Brooks *et al.*, 2005; Chu and Metcalfe, 2007; Ramirez *et al.*, 2009; Schultz and Furlong, 2008; Schultz *et al.*, 2010), and surveys in US have reported levels of 12–540 ng/L of fluoxetine in surface waters and effluents (Kolpin *et al.*, 2002). 4-Nonylphenol is used as an industrial detergent or as a co-adjuvant for many pesticide formulations and is present in great amounts in surface waters, reaching levels as high as 8 µg/L in surface Spanish waters (Navarro *et al.*, 2009). It is also produced by the degradation of industrial surfactants, like nonylphenol ethoxylates and carboxylates. Fluoxetine is known to increase reproduction in *Daphnia* (Flaherty and Dodson, 2005). Despite that fluvoxamine reproductive effects have not been tested in *D. magna*, it is ten-fold more active than fluoxetine inducing spawning in zebra mussel (Fong, 1998). 4-Nonylphenol is known to act as a weak estrogenic compound in vertebrates but its ecotoxicological mode of action in *Daphnia* is uncertain (Barata *et al.*, 2004). Indeed recent studies indicate that 4-nonylphenol may inhibit, unchange or enhance off-spring production in *D. magna* (Baldwin *et al.*, 1997; Brennan *et al.*, 2006; LeBlanc *et al.*, 2000b).

2.2. Material and methods

2.2.1. Chemicals

The substances employed in toxicity experiments were: 4-nonylphenol (CAS No 104-40-5, PESTANAL® analytical grade standard, 98.4% purity, Riedel-de-Haen, Germany); fluoxetine hydrochloride (CAS-No 56296-78-7; analytical standard, purity 100% Sigma-Aldrich, USA), and fluvoxamine maleate (CAS-No

61718-82-9, analytical standard, purity 100%, Sigma–Aldrich, Netherlands). All other chemicals were analytical grade and were obtained from Merck (Germany).

2.2.2. Experimental animals

Two clones of *D. magna* were selected. Two laboratory clones hereafter referred as T, cultured in the laboratory since 1999 (Barata *et al.*, 2006), and clone F, which has been the subject of many investigations (Barata and Baird, 2000). Clones T and F are characterized by having low and high reproductive outputs under laboratory conditions, respectively. For each clone, individual or bulk cultures of 10 animals/L were maintained in ASTM hard synthetic water (ASTM, 1995) as described by Barata and Baird, 2000. Individual or bulk cultures were fed daily with *Chorella vulgaris* Beijerinck (5×10^5 or 10^6 cells/mL, respectively, corresponding to 1.8 or 3.6 µg C/mL (Barata and Baird, 1998). The culture medium was changed every other day, and neonates were removed within 24 h. Photoperiod was set to 14h light: 10h dark cycle and temperature at $20 \pm 1^\circ\text{C}$.

2.2.3. Experimental design

Five distinct reproduction tests were conducted over one year to study clonal, stage- and food-dependent contaminant effects on growth and reproduction traits. Experiments included three life-table and two adult reproduction experiments that followed OECD guidelines (OECD, 1981) and (Barata and Baird, 2000) procedures.

2.2.3.1. Life-table tests

Life-table tests allowed the determination of the effects of the studied compounds on growth, reproduction and on population growth rates of juveniles exposed from birth until their fourth brood. In the first two life-table experiments, neonates (<24h old) of clones T and F were exposed until their fourth brood (approximately 21–23 days at 20°C) to 10, 20, 40, 60, 80, 110 µg/L of 4-nonylphenol; 10, 40, 80 µg/L of fluoxetine and 3, 7 and 30 µg/L of fluvoxamine, at the food ration of 5×10^5 cells/mL of *C. vulgaris*. In the third one, neonates (<24h old) of clone F were exposed until their fourth brood to 15, 40 and 7 µg/L of 4-

nonylphenol, fluoxetine and fluvoxamine, respectively, at three food rations (1.6×10^5 , 3.3×10^5 and 6×10^5 cells/mL of *C. vulgaris*; referred as low, intermediate and high, respectively). For each individual, its survival, the number of maturation instars, age, body length and the size of each clutch were monitored. In the third and fourth brood, the body length and sex of at least 10 neonates were also considered. Body length measurements were performed from the head to the base of the spine using a Nikon stereoscope microscope (SMZ 150, Nikon, Barcelona, Spain) and the ImageJ software (<http://rsb.info.nih.gov/ij/>). The intrinsic rate of increase (r) was computed iteratively from the Lotka equation (eq. 1.1)

$$\sum_{x=0}^{\infty} e^{-rx} l_x m_x = 1b \quad (\text{eq. 1.1})$$

where l_x is the proportion of the females surviving to age x (days) and m_x is the number of juveniles produced per surviving female between the ages x and $x+1$. The age at birth was set to 0. The 95% confidence intervals were estimated by the Jackknife method (Meyer *et al.*, 1986).

2.2.3.2. Adult reproduction tests

Adult reproduction tests allowed the determination of the effects of pollutants on growth and reproduction rates on adult stages. In two consecutive experiments, nine to ten gravid females of clones T and F were separately exposed to the same concentrations of 4-nonylphenol, fluoxetine and fluvoxamine described for life-table tests using a food ration of 5×10^5 cells/mL of *C. vulgaris*. Experiments started with 8–9-day-old gravid females, which were exposed during three consecutive broods to the studied chemicals (10–14 days). Cultures of 100–150 individuals (<24h old neonates) were initiated and maintained in bulk cultures as described above. Within 24h of deposition of the first clutch into the brood chamber, single females were removed and randomly assigned to each treatment. The first batch of neonates (hatching within the first 48–72h) was always discarded and not evaluated, as these animals were not exposed to the tested chemicals during their entire developmental period (Barata and Baird, 2000). Thus, only neonates from the second, third and fourth broods were counted for assessing effects on total offspring production. The

body length of each individual female and of 10 neonates released in the third and fourth brood were also measured to assess effects on growth and on reproductive investment per offspring, respectively. Neonate sex was also

$$n \ p_j^{t_j} \lambda^{-t_j} + p_a \ \lambda^{-1} = 1 \quad (\text{eq. 2.2})$$

accounted for.

Contributions of the measured fecundity rates on population growth rates $\lambda=e^r$ were also evaluated using the simplified 2-stage model of (Sibly *et al.*, 2000) (eq. 2.2) that assumes age-independent birth (n), juvenile (p_j) and adult (p_a) mortality rates.

Selected parameters were as follow: p_j and p_a were set to 1 since no mortality occurred during the experiments; age at first reproduction (t_j) was similar within experiments and was set to 11 and 10.5 days for clones T and F, respectively. The number of offspring produced daily per female (n) was determined for each female considering the offspring produced in the second, third and four clutches, during a time period of 8 days. A time unit of 1 day was employed for all treatments because reproductive output was measured every day. The 95% confidence intervals were estimated by the Jackknife method (Meyer *et al.*, 1986).

2.2.3.3. Test solutions

In all five experiments, test solutions were prepared by adding appropriate amounts of a concentrated stock solution to ASTM hard water, mixing thoroughly. Acetone (HPLC grade; <0.1mL/L) was used as carrier solvent. To remove any potential confounding effect, the same concentration of acetone was added to all treatments, including the solvent control. A control without acetone was also used to assess the possibility of carrier effects. Individuals were maintained in 150mL of ASTM hard water, in 200mL screw top glass jars, with the addition of a standard organic extract (Barata and Baird, 2000). Exposure solutions with algae were changed every day.

2.2.4. Chemical water analyses

Duplicated water samples of freshly prepared and old (24h) test solutions were collected at the beginning and end of the tests to determine that measured

oxygen levels and pH were within the limit established by OECD guidelines (OECD, 1981). Dissolved oxygen concentration (DO) was measured using a polarographic oxygen electrode coupled to a CyberScan DO 300/3001 EUTECH model meter (Lab Process Distributions, Calella, Barcelona, Spain). The pH was determined using an epoxy-body combination electrode, coupled to a Crison micro pH 2001 meter and calibrated with standard pH buffer solutions (Sigma, Madrid, Spain). In all tests, oxygen levels were within 90% of air saturation and pH values varied between 7.5 and 8. Stability of the compounds during the test was confirmed using HPLC. Analysis of 4-nonylphenol, fluoxetine and fluvoxamine was restricted to two or three concentrations of final test solutions (24h). Selected exposure levels included 15, 20 and 40µg/L of 4-nonylphenol; 10, 40 and 80µg/L for fluoxetine and 7 and 30µg/L of fluvoxamine. For the analysis of 4-nonylphenol, Oasis 200mg cartridges were conditioned with 10mL of dichloromethane, 10mL of methanol and 10mL of water and after pre-concentration, elution was performed with 10mL of acetone: dichloromethane (1:1). This extract was then reduced under nitrogen to incipient dryness and reconstituted with 500µL of ethyl acetate. Analysis was performed by gas chromatography coupled to mass spectrometry (GC–MS) on a Thermo Finnigan Trace GC/MS 2000 Series (i.e. low resolution MS) with a 30m HP-5-MS, 0.25mm ID, 0.25µm film thickness 220 column (Agilent, ref. 19091S-433), following the gradient reported in a previous study where 4-nonylphenol eluted at 12.44 min (Fernández-Sanjuan *et al.*, 2009). For the pre-concentration of fluoxetine and fluvoxamine, Oasis 60mg SPE cartridges were conditioned with 10mL of methanol followed by 10mL of water. A volume of 100mL of water were pre-concentrated at a flow rate of 10mL/min and eluted with 2×4 mL of methanol. This extract was then reduced under nitrogen to incipient dryness and reconstituted with 500µL of the mobile phase. Fluoxetine and fluvoxamine were analysed by high performance liquid chromatography coupled to diode array detection (HPLC–DAD) with an Agilent Technologies 1200 series and detector DAD G1315D. A volume of 50µL of extract was injected on a 50×4.6mm XBridge TM C18 column of 3.5µm of particle diameter (Waters, USA). A gradient elution starting at 70:30 water (pH 4.0)–acetonitrile to 100% acetonitrile in 20 min was used at a flow rate of 0.8mL/min. Acquisition was done at a wavelength of 226 and 252nm. Under those conditions, fluvoxamine

and fluoxetine eluted at 3.56 and 4.15 min, respectively. Recoveries (mean \pm SE) of 4-nonylphenol, fluoxetine and fluvoxamine from water samples were determined using external standard quantification.

2.2.5. Data analyses

Each experiment was analysed separately to exclude confounding effects of inter-trial variation (Barata and Baird, 1998). In the three life-table experiments, not all individuals invested the same number of developmental instars to maturity (Table 2.2), thus maturation instar was also considered as a fixed factor in the ANOVA analyses to further differentiate treatment from non-treatment (maturation instar) effects on life-history responses. However, some of the treatments showed no variation in maturation instar (Table 2.2), and a full factorial ANOVA design including maturation instar interactions was not possible. To overcome problems in the interpretation of interactions obtained from multi-factorial designs with missing cells (Sokal and Rohlf, 1995), treatment versus maturation instar or treatment versus maturation instar versus food ration interactions were not considered. When significant ($P<0.05$) treatments effects occurred, post-hoc Dunnet's tests were performed to compare exposure treatments with solvent controls. In the adult reproduction tests, differences across solvent control and the rest of treatments in life-history responses were compared using one way ANOVA followed by Dunnet's multiple comparison tests. Prior to analysis, all data except age at first reproduction were log transformed to improve normality and variance homoscedasticity (Sokal and Rohlf, 1995). Effects of treatment and maturation instar in age at first reproduction were analysed by non-parametric Kruskal-Wallis and Mann-Whitney tests, respectively. Analyses of r were based on Jackknife pseudo-values (Meyer *et al.*, 1986) obtained using the full data set; thus it was not possible to differentiate between treatment and maturation instar effects. To further investigate contributions of maturation instar on r responses, for each clone or food ration, the proportion of females maturing in five or six instars was computed and maturation instar Jackknife pseudo-values were compared using Student's t or ANOVA tests. Tests were conducted with the aid of the statistical package IBM SPSS v19 and General Linear Models were used

instead of conventional ANOVA when more than two factors or unequal replicates across treatments were considered.

2.3. Results

2.3.1. Contaminant analyses

Nominal versus measured test concentrations of the studied compounds are depicted in Table 2.1.

Table 2.1. Nominal and measured chemical concentrations (Mean and SD) in 24h old tests solutions. Results for duplicated samples taken in each tests are polluted. N, sample size.

Chemical	Nominal	N	Measured	
			Mean	SD
4 Nonylphenol	15	2	12.9	1.2
	20	8	17.4	1.8
	40	8	36.0	2.7
Fluoxetine	10	10	9.5	0.3
	40	8	38.7	2.4
	80	8	79.5	4.8
Fluvoxamine	7	10	6.3	0.4
	30	8	28.6	1.8

Differences in sample size are due to the fact that not all exposure levels measured were used in all experiments (i.e. 15µg/L of 4-nonylphenol was only used in the life-table food test). Mean recoveries of 4-nonylphenol were 88%, those of fluoxetine were 97% and 93.5% for fluvoxamine. For clarity, all results are depicted in nominal concentrations.

2.3.2. Life-table tests (clone effects)

High 4-nonylphenol levels impaired significantly the survival of exposed individuals and embryos in life-table experiments ($P<0.05$, Wilcoxon–Gehan tests). All individuals died before reproduction at 110µg/L and, in clone F, only few individuals reproduced at 60 and 80µg/L (Figure 2.1). Embryo survival, measured as the percentage of aborted eggs, was also severely impaired at 60 and 80µg/L in clone F (Figure 2.2). These treatments were excluded from the

estimation of total offspring production per female since only females that release fourth clutches fully alive were considered.

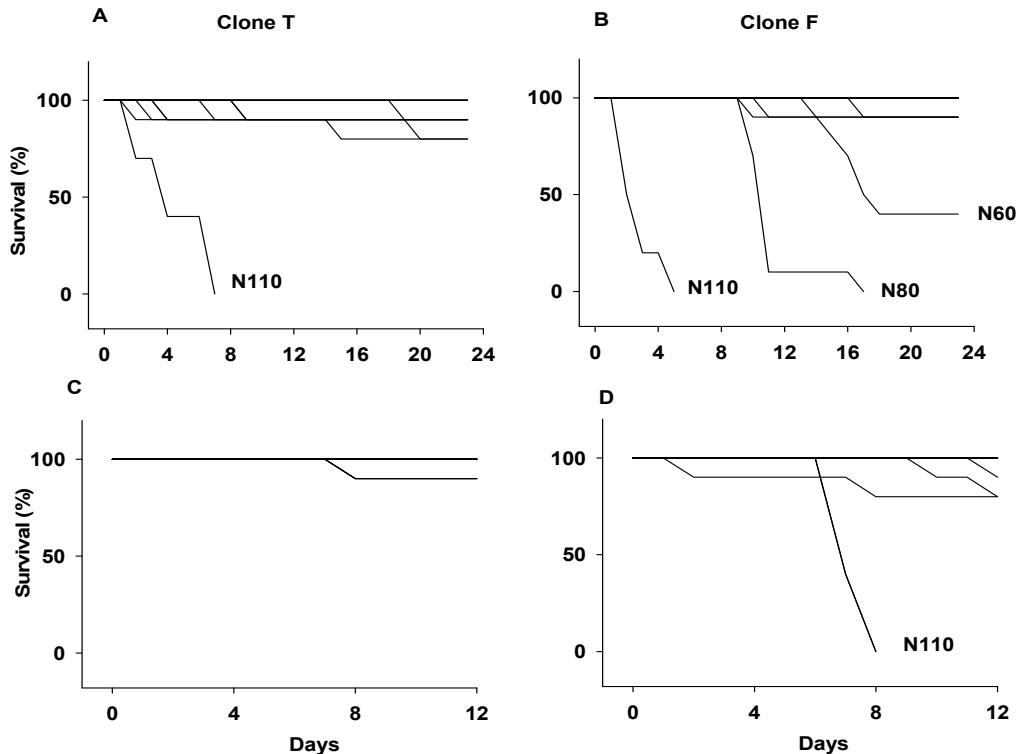


Figure 2.1. Survival curves of *D. magna* individuals from clones T (A, C) and F (B, D) exposed to the studied compounds in the life-table (A, B) and adult reproduction tests (C,D). Only those treatments with significant mortality ($P<0.05$, based on Wilcoxon-Gehan tests) were identified. N60, N80, N110 are 60, 80, 110 μ g/l of 4-nonylphenol, respectively.

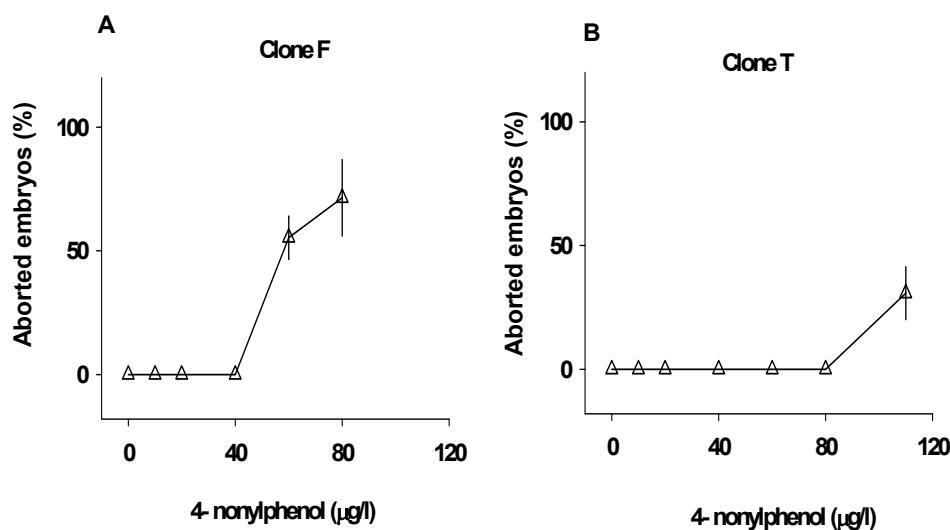


Figure 2.2. Percentage (Mean \pm SE, $N=8-10$) of aborted embryos release by *D. magna* females in life-table (A) and adult reproduction tests (B) from clones F and T, respectively.

Treatment and maturation instar effects on the selected life-history traits depicted in Figure 2.3 are indicated in Tables 2.2 and 2.3. In experiment 1 and 2 both clones had the same percentage of females investing six developmental instars until maturity (there were no significant differences ($P < 0.05$, Chi square tests; Table 2.2).

Table 2.2. Percentage of females taking six instars to mature in the life-table experiments performed between clones and across food levels. Chi square results testing for differences across treatments are also depicted. C, SC, L, M, H are control, solvent control treatments, Low, intermediate and high food level ratios, respectively. NP, Fx, Fv are 4-nonylphenol, fluoxetine, fluvoxamine treatments, respectively, and the numbers after those names are the exposure levels. ns $P > 0.05$; *** $P < 0.01$

Exposure	Exp1		Exp2		Exp 3		
	Clone T	Clone F		Food	Exposure	Clone F	
C	55.6	90		L	C	22.2	
SC	60	60			SC	33.3	
NP10	55.6	90			NP15	100	
NP20	100	60			Fx 40	80	
NP40	70	70			Fv 7	55.6	
NP60	90	70	M		C	16.7	
NP80	80	70			SC	60	
Fx 10	100	80			NP15	57.1	
Fx 40	100	40			Fx 40	30	
Fx 80	88.9	70			Fv 7	33.3	
Fv 3	66.7	80	H		C	10	
Fv 7	90	80			SC	10	
Fv 30	66.7	88.9			NP15	20	
					Fx 40	50	
					Fv 7	10	
Chi-square	19.6 ns		12.6 ns			33.2 ***	

Maturation instar effects on the studied life-history traits were evident for age at first reproduction with females investing an additional instar reproducing later. Only in clone F, maturation instar also affected significantly ($P < 0.05$) female body length and offspring production, with females maturing later being larger and producing more offspring than those maturing earlier. Interestingly, females maturing earlier in clone T had also higher population growth rates than those whose maturity was delayed. Significant ($P < 0.05$) treatment effects were observed for all traits but female body length at the fourth brood. In clone T there was significant ($P < 0.05$) treatment effects on age at first reproduction ($P < 0.05$, Kruskal–Wallis tests) since females exposed to fluvoxamine matured earlier than those of controls (Fig. 2.3).

Table 2.3. ANOVA analysis results (degrees of freedom, df; Fisher's ratio, F; and significant levels, Sig.) testing maturation instar (MI) and treatment (T) effects on life history traits responses of clones T and F in life-table tests. 1st, at first reproduction; 4th, at the fourth brood

	Effect	df	F	Sig.		df	F	Sig.
Life Table test (Exp1)					Life Table test (Exp 2)			
Clone T					Clone F			
Age α	T	12	32.6	<0.01	Age α	T	12	10.3 0.59
	MI	1	194.0	<0.02		MI	1	739.0 <0.01
Body length 1st ρ	T	12,107	0.9	0.58	Body length 1st ρ	12,114	2.7	<0.01
	MI	1,107	1.3	0.26		1,114	587.0	<0.01
Clutch size 1st ρ	T	12,107	2.1	0.026	Clutch size 1st ρ	T	12,107	19.9 <0.01
	MI	1,107	2.3	0.13		MI	1,108	30.1 <0.01
Body length 4th ρ	T	12,106	0.9	0.52	Body length 4th ρ	T	11,97	0.8 0.67
	MI	1,106	0.0	0.89		MI	1,97	26.4 <0.01
Total Offspring ρ	T	12,100	3.0	<0.01	Total Offspring ρ	T	10,94	10.9 <0.01
	MI	1,100	1.9	0.17		MI	1,94	15.7 <0.01
Offspring size ρ	T	12,208	16.9	<0.01	Offspring size ρ	T	11,189	22.1 .000
	MI	1,208	11.3	<0.01		MI	1,189	3.9 .050
$r\gamma$	T	12,110	1.1	0.41	$r\gamma$	T	12,109	8.9 <0.01
	MI	121	8.3	<0.01		MI	120	0.1 0.90

α Non parametric Kruskal-Wallis and Mann-Whitman tests; ρ two way ANOVA tests without interactions terms; γ , separate one way ANOVA tests

Females of clone T exposed to 20 and 40 μ g/L of 4-nonylphenol produced more offspring than solvent and control treatments. In clone F, females exposed to 10 and 40 μ g/L of 4-nonylphenol released more and less offspring than controls, respectively. Neither SSRIs nor 4-nonylphenol affected offspring sex (all neonates were females). In both clones, females exposed to high 4-nonylphenol levels produced smaller offspring than those of solvent controls. Population growth rates were only significantly ($P<0.05$) affected by exposure treatment in clone F, with females exposed to 40, 60 and 80 μ g/L of 4-nonylphenol having lower values than solvent controls.

2.3.3. Life-table tests (food effects)

Food ration alone, treatment or its interaction and/or maturation instar affected all the studied life-history traits. Survival of individuals exposed to 4-nonylphenol was significantly affected at low and intermediate food ($P<0.05$; Wilcoxon–Gehan test; Fig 2.3). There were significant differences ($P<0.05$, Chi square tests) in the percentage of females investing six developmental instars until maturity across food levels (Table 2.2). Increasing food rations decreased the number of females maturing in six instars (Table 2.2).

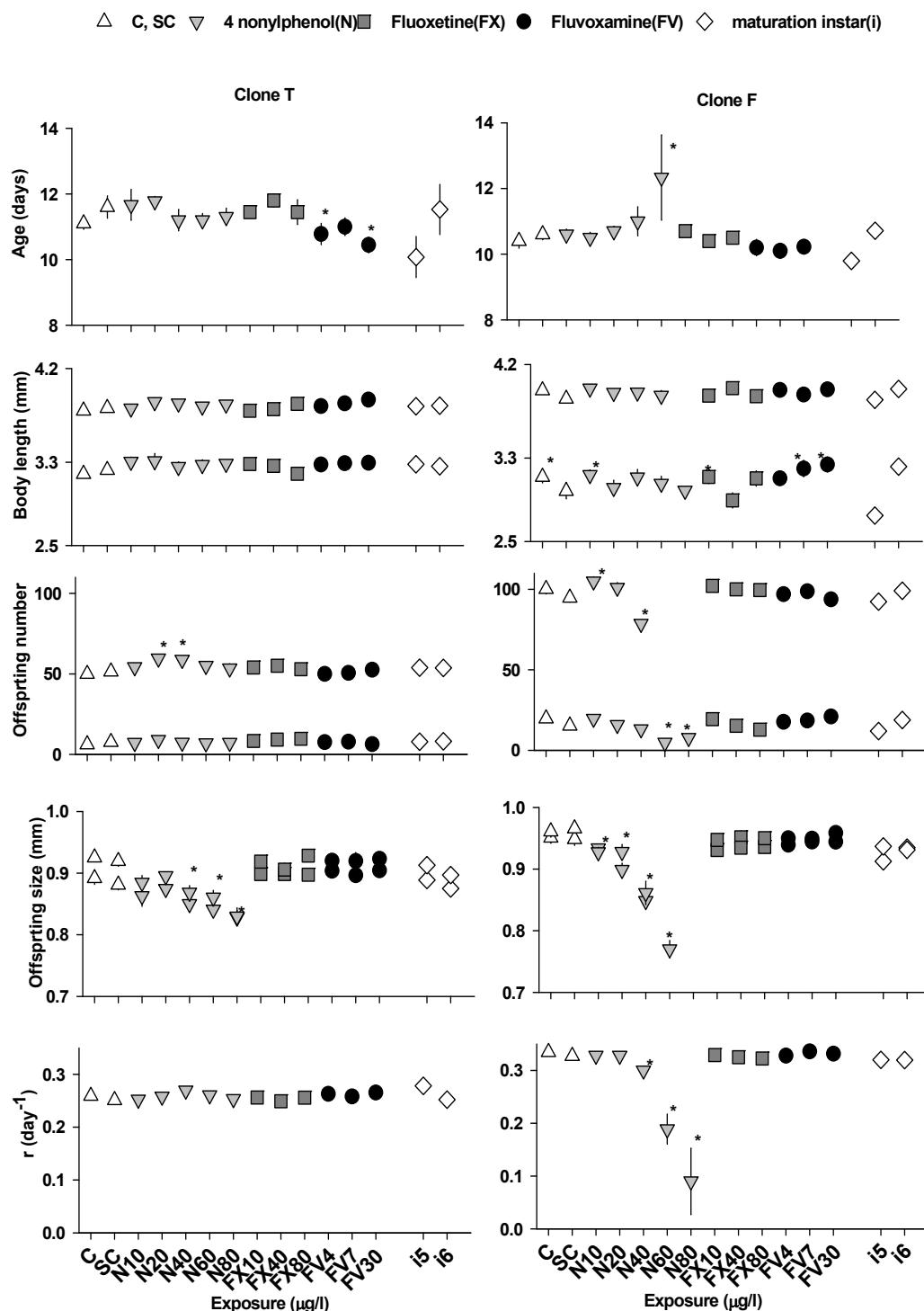


Figure. 2.3. Life history responses (Mean±SE, N=8-10) of *D. magna* individuals from clones T and F exposed to the studied compounds from birth to the fourth brood. Each column of graph panel corresponds to one clone. Body length graphs include the length of first and fourth brood females; those of offspring number, the values for first clutch and total offspring, and those of offspring size of neonates from third and fourth broods. * Significant ($P<0.05$) differences from solvent controls following ANOVA and Dunnet's tests. C, SC; NP, FX, FV are Control, Solvent control, 4-nonylphenol, fluoxetine and fluvoxamine treatments, respectively; r , intrinsic rate of population growth. For chemical treatments the numbers identify exposure levels.

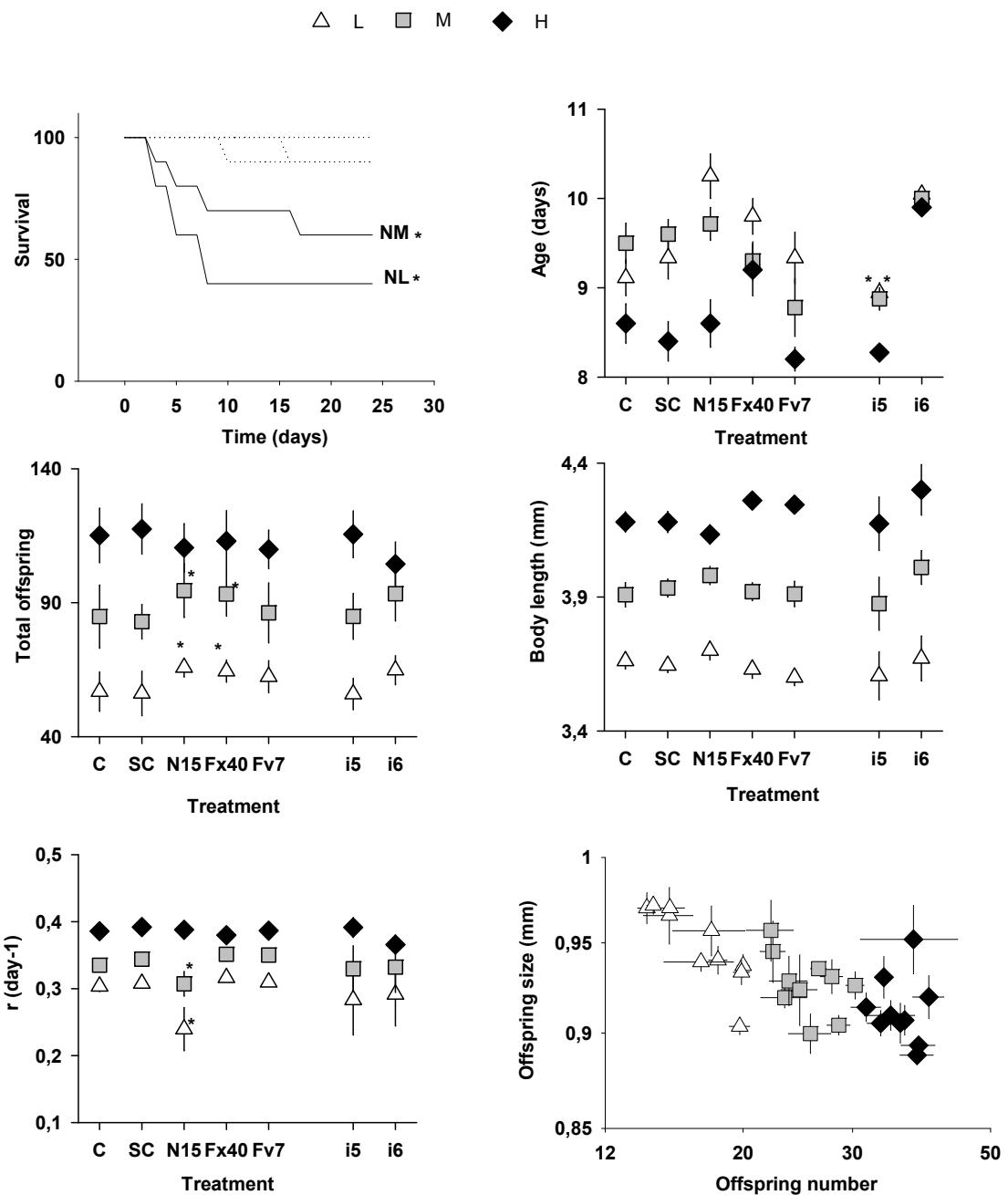


Figure 2.4. Life history responses (Mean±SE, N=10) of *D. magna* individuals from clone F exposed to the studied compounds from birth to the fourth brood at three different food level ratios (Low, L; intermediate, M; and high, H). In the Survival graphs only 4-nonylphenol treatments at low and intermediate food levels (NL, NM, respectively) with significant ($P<0.05$) mortality were identified. In the remained graphs significant ($P<0.05$) differences from solvent controls within food levels following ANOVA and Dunnet's tests are indicated with an asterisks. C, SC; N15, Fx40, Fv 7 are control, solvent control, 15 μ g/l of 4-nonylphenol, 40 μ g/l of fluoxetine and 7 μ g/l of fluvoxamine, respectively. r , intrinsic rate of population growth.

Table 2.4. ANOVA analysis results (degrees of freedom. df; Fisher's ratio. F; and significant levels. Sig.) testing maturation instar (MI). Treatment (T) and food level (F) effects on life history traits responses of clone F in the food life-table test (Exp. 3).

	Effect	df	F	Sig.
Age α	F	2	28.9	<0.01
	T	4	10.3	0.036
	MI	1	140.	<0.01
	FxT	14	49.5	<0.02
Total Offspring ρ	F	2.102	325.	<0.01
	T	4.102	3.7	<0.01
	MI	1.102	2.0	0.156
	FxT	2.102	12.7	<0.01
	F x MI	8.102	1.1	0.389
	T x MI	4.102	2.5	0.048
Body length 4th ρ	F	2.106	320.	<0.01
	T	4.106	0.4	0.798
	MI	1.106	31.0	<0.01
	FxT	2.106	0.6	0.539
	F x MI	8.106	1.9	0.071
	T x MI	4.106	0.7	0.619
Offspring size ϵ	Fec	1.85	14.7	<0.01
	F	2.85	3.0	0.060
	T	4.85	1.1	0.375
	MI	1.85	9.6	<0.01
	FxT	2.85	3.0	0.060
	F x MI	8.85	2.2	0.034
	T x MI	4.85	0.2	0.935
$r \gamma_1$	F	2.126	66.6	<0.01
	T	4.126	9.0	<0.01
	F x T	8.126	3.6	<0.01
$r \gamma_2$	F	2.135	49.2	<0.01
	MI	1.135	0.2	0.688
	F x MI	2.135	1.4	0.238

α Non parametric Kruskal-Wallis and Mann-Whitman tests; ρ three way ANOVA tests without three factor interactions terms; ϵ . three way ANCOVA results considering clutch size (Fec) as covariate. γ Separate two way ANOVAS testing for Food and treatment (γ_1) or food and maturation instar (γ_2) effects

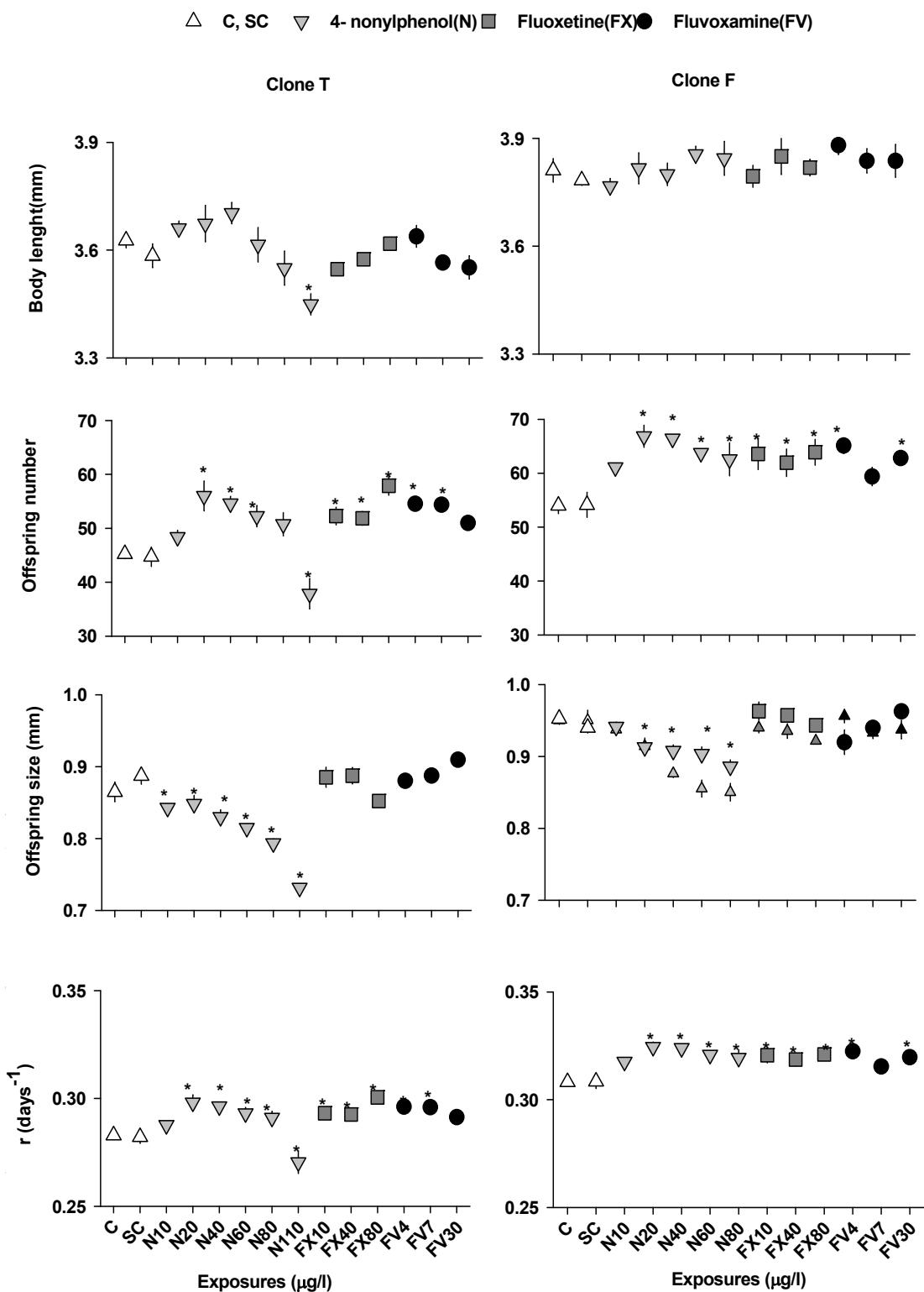


Figure 2.5. Life history responses (Mean \pm SE, N=10) of *D. magna* gravid females exposed to the studied compounds during three consecutive broods. * significant ($P<0.05$) differences from solvent controls following one way ANOVA and Dunnet's tests. C, SC; NP, FX, FV are Control, Solvent control, 4-nonylphenol, fluoxetine and fluvoxamine treatments, respectively; r, intrinsic rate of population growth. For chemical treatments the numbers identify exposure levels.

Also, increasing food rations increased significantly ($P<0.05$, table 2.4, Fig. 2.4) total offspring production, final body length and population growth rates and decreased age at first reproduction and offspring size. Significant ($P<0.05$, Table 2.4) maturation instar effects on the studied life-history traits were evident for age at maturity and body length of fourth brood females. As a result individuals investing an additional instar were larger and start reproduction later (Figure 2.3) significant ($P<0.05$) treatment and treatment by food interaction effects were also observed for total offspring production (Table 2.4) with females exposed to 4-nonylphenol and fluoxetine producing more offspring only at low and intermediate food rations(Fig. 2.4). Within a food ration, population growth rates were similar across treatments except for those individuals exposed to 4-nonylphenol at low and intermediate food rations, that were lower (Table 2.4, Fig. 2.4). The sex of offspring was unaffected by treatment, and offspring size was inversely related to offspring number irrespectively of food ration and exposure treatment (Table 2.4, Fig. 2.5).

2.3.4. Adult reproduction tests

At $110\mu\text{g/L}$ of 4-nonylphenol, no adult females from clone F survive long enough to reproduce and substantial embryo mortality (up to 30%) occurred within females of clone T (Fig 2.1). Statistical results obtained from the reproduction experiment performed on adult females of clones T and F, denoted mainly significant ($P<0.05$) treatment effects on both the number and size of offspring (Fig. 2.5). The final body length of all females tested was similar for all treatments, except for daphnids of clone T exposed to $110\mu\text{g/L}$ of 4-nonylphenol, which were smaller. Total offspring increased in 9 out of the 12 exposure treatments for both clones, relative to solvent controls. Treatments did not affect offspring sex and the size of neonates decrease significantly ($P<0.05$) with increasing 4-nonylphenol exposure levels. Observed differences in reproduction rates mirrored in significant population growth rate differences between exposed and non-exposed adult females.

2.4. Discussion

The results obtained in this study indicated different modes of action of the studied chemicals when individuals were exposed from birth or during adulthood. Life-history effects also varied at low and high food rations. Juveniles and adults of clone T were more tolerant to 4-nonylphenol than those of clone F. When exposed during both juvenile and adult stages in the typical standardized 21 days reproduction assay, the number of maturation instars taken to maturity together with exposure treatments, affected differently growth- and reproductive-related traits in the two clones studied. Thus, it was not always possible to differentiate maturation instar effects from those caused by the studied chemicals, since individuals did not take either five or six instars to mature in all treatments. Females that invested an additional instar to mature were older at first reproduction and in clone F were also larger in size and produced more offspring than those maturing in five instars. When maturation instar effects were accounted for, the most important contaminant effects were as follows: fluvoxamine increased juvenile developmental rates relative to control treatments (i.e. females reproduced earlier) and females exposed to low levels of 4-nonylphenol (20, 40µg/L for clone T; 10µg/L for clone F) produced more but smaller offspring than control treatments. The previous life-history pollutant effects, however, did not translate into higher population growth rates. In clone F, reduced offspring production and population growth rates of females exposed to 40, 60 and 80µg/L of 4-nonylphenol were likely related to general toxic effects, since at 60 and 80µg/L severe effects on adult and embryo survival were observed. Thus, 4-nonylphenol caused a bi-phasic response enhancing offspring production at low concentrations and impairing survival, growth and reproduction at high concentrations. The size and number of offspring and age at first reproduction are strongly affected by food rations in *D. magna* (Barata and Baird, 1998; Barata *et al.*, 2001). *D. magna* females growth less, reproduce latter, produced fewer but larger offspring and have lower population growth rates at low than at high food rations. This is a well-known adaptive strategy that allows *D. magna* to regulate its growth and per offspring reproductive investment, to maximize its fitness under variable food conditions. In the present study, the third life-table experiment was specifically designed to

assess how food rations could affect the observed life-history responses of individuals from clone F across the studied pollutants. As expected, food affected all the studied traits with females maturing earlier, being bigger and producing more and smaller offspring at high food rations. The availability of food also affected pollutant effects on the studied life-history traits. Survival of individuals exposed to 15 μ g/L of 4-nonylphenol decreased dramatically at the low food ration. These findings agree with recent results indicating that low and realistic exposure levels of 4-nonylphenol (10 μ g/L) impaired *D. magna* survival at low food rations (Beklioglu *et al.*, 2010). Only at low and intermediate food rations, fluvoxamine increased juvenile developmental rates and 4-nonylphenol and fluoxetine increased offspring production relatively to control treatments. When the previous life-history changes were computed to estimate population growth rates, only those 4-nonylphenol treatments that showed high mortality rates had lower population growth rates than control treatments. Interestingly, a change just as little as two fold in food rations, within the range of 3.3×10^5 and 6×10^5 cells/mL of *C. vulgaris*, affected dramatically the observed pollutant effects. Thus, slight variations in food rations could explain the reported discrepancies in the effects of 4-nonylphenol and fluoxetine on *D. magna* reproduction responses in 21 days chronic assays (Baldwin *et al.*, 1997; Comber *et al.*, 1993; Flaherty and Dodson, 2005; Hansen *et al.*, 2008). Such small variations on food rations are very difficult to control experimentally and may have major effects on life-history responses (Barata and Baird, 1998). Another important finding of the food life-table experiment was that neither food nor pollutant levels affected the inverse relationship between offspring size and clutch size. This means that 4-nonylphenol and fluoxetine at low doses (15 and 40 μ g/L, respectively) increased offspring production without affecting per offspring reproductive investment (i.e. neonate size). This finding, however, did not occur at higher 4-nonylphenol doses (adult reproduction tests with clones T and F), where females produced smaller offspring than controls. In summary, life-table experiments showed negligible population growth effects associated to the increased juvenile developmental rates or offspring production induced by pollutants. Conversely, concentrations high enough to impair offspring production and/or adult and embryo survival did affect negatively population growth rates. Nevertheless, and according to previous studies (Boersma, 1997;

Hammers-Wirtz and Ratte, 2000), the offspring produced under 4-nonylphenol exposures should be less fitted than those produced under control and SSRIs, because of their smaller size. This prediction agrees with the observation that second generation females were more sensitive to 4-nonylphenol than first generation ones (Brennan *et al.*, 2006). Exposure of adults to the studied chemicals increased total offspring production and population growth rates, with the only exception of females exposed to 110 μ g/L of 4-nonylphenol. Interestingly, per offspring reproductive investment (neonate body length) decreased in a concentration related manner in 4-nonylphenol treatments but not under exposure to fluoxetine and fluvoxamine, a result compatible with the data from life-table experiments. Responses to 4-nonylphenol agree with the “Principle of Allocation” that states that the amount of energy an individual can invest in maintenance, growth and reproduction is limited within a given energy input (Sibly and Calow, 1986). Under 4-nonylphenol exposures, females produced more but smaller offspring thus maintained or even reduced total reproductive investment and the gains on current fitness in terms of population growth rates of exposed adult females was counteracted by reduced offspring fitness (smaller offspring). Under fluoxetine and fluvoxamine exposures, however, females produced more offspring of apparently similar size to control treatments. This means that there was not a clear fitness pay-off of producing more offspring. Enhanced offspring production and altered per offspring reproductive investment and embryo toxicity of females exposed to 4-nonylphenol have been previously reported (LeBlanc *et al.*, 2000b). Similarly, increased fecundity of females exposed to fluoxetine has also been reported for in *D. magna* and *Ceriodaphnia dubia* (Brooks *et al.*, 2003a; Flaherty and Dodson, 2005). With our current knowledge, it is difficult to provide a plausible hypothesis explaining the observed differences on life-history responses between life-table and adult tests, across food levels and between 4-nonylphenol and SSRIs. One possible physiological explanation is that the studied SSRIs acted altering the activity of serotonin in *D. magna* by inhibiting the uptake of this chemical at the receptor like in mammalian species. Consistent with this hypothesis, sublethal concentrations of SSRIs have been found to alter the reproductive physiology of *D. magna* and other invertebrates (Brooks *et al.*, 2003a; Flaherty and Dodson, 2005; Hansen *et al.*, 2008). In

molluscs, SSRIs alterations were associated with increased levels of serotonin (Fong, 1998). The increase of SSRI effects on juvenile developmental rates and offspring production at low food rations, as found in our study, indicates that these animals might reduce their production of serotonin at low food rations, whereas at high food rations, serotonin levels might be maximal. As a result SSRIs increased serotonin activity at low food but not at high food rations where serotonergic receptors may be already saturated. This hypothesis reflects the main pharmacological role of SSRIs in mammals, as they block the presynaptic membrane serotonin transporter, thereby inhibiting synaptic reuptake and recycling of serotonin (Wong *et al.*, 1995). In addition to its anti-depressive effects, chronic exposures to SSRIs may also induce desensitization of serotonergic receptors (Le Poul *et al.*, 2000; Vidal *et al.*, 2009). If translated to *D. magna*, a similar effect could explain the results from juveniles exposed to SSRIs in life-table tests, thus making adult stages insensitive to SSRIs exposures. Although the regulatory role of serotonin in controlling *D. magna* growth and reproduction is not known, there is increasing evidence that serotonin/biogenic amines are present and might regulate fecundity in crustaceans (Barthélémy *et al.*, 2006; Bellon-Humbert and Van Herp, 1988; Mazurová *et al.*, 2008; Wongprasert *et al.*, 2006). According to LeBlanc *et al.* (2000b) the increase in offspring production and the decrease in neonate size and embryo viability induced by 4-nonylphenol, may indicate that this compound stimulates egg production without increasing some critical developmental component provided to the eggs by the maternal organisms, such as ecdysteroids, essential fatty acids or triglycerides. Alternatively, LeBlanc *et al.* (2000b) also stated that the metabolic effects of 4-nonylphenol characterized previously by Baldwin *et al.* (1997) using testosterone as a substrate, might also be relevant to endogenous substrates (such as ecdysone), resulting in perturbations in their provision to the newly produced eggs as well as in adults. Ecdysteroids play a critical role in developing embryos (Subramoniam, 2000). A close correlation between haemolymph ecdysteroid levels and ovarian maturation and vitellogenin synthesis, has been reported in some crustacean species (Okumura, 1992). A recent study showed that 4-nonylphenol was able to disrupt ecdysone levels in the mysid crustacean *Americamysis bahia* during the intermolt period (Hirano *et al.*, 2009) and as a result disrupted growth.

Therefore, both hypotheses are equally valid to explain at least part of the observed reproductive features of 4-nonylphenol. Nevertheless, both 4-nonylphenol and SSRIs may interact with multiple targets in mammals and as such are likely to do so also in *D. magna*. Therefore, future investigations need to be performed to elucidate the mechanisms of action of these chemicals in *D. magna* and other aquatic organism. In summary, the results reported in this study indicate that the three studied substances were able to increase offspring production and/or juvenile developmental rates similarly across the studied clones and that exposure stage scenarios, and food rations modified the observed responses. For both, SSRIs and 4-nonylphenol, observed effects occurred at low concentration levels (3–15µg/L). Surveys in US have reported levels of 12–540ng/L of fluoxetine in surface waters and effluents (Kolpin *et al.*, 2002) but total concentrations of SSRIs in aquatic systems were measured in the range of 840ng/L (Vasskog *et al.*, 2008) to 3.2µg/L (Metcalfe *et al.*, 2010). 4-Nonylphenol levels in surface waters as high as 8µg/L have been reported in surface waters (Navarro *et al.*, 2009). Therefore, the observed responses measured in this present study occurred at realistic field exposure levels. Moreover, in real field situations the occurrence of SSRI mixtures (Christensen *et al.*, 2007) of different enantiomers and metabolites and acidic and basic water conditions are likely to affect dramatically bio-availability and toxicity of SSRIs to aquatic organisms (Oakes *et al.*, 2010). Fluoxetine and other SSRIs are weak bases, which ionize in water at ambient pHs (Oakes *et al.*, 2010). Ionized forms are more soluble in water than non-ionized ones and, hence, should be more toxic and bio-available to organisms. This is the case for fluoxetine, its major metabolite norfluoxetine and other SSRIs as reported by Nakamura *et al.* (2008) and Valenti *et al.* (2009) in fish. Enantiomer and major metabolite specific effects of SSRIs should also be considered, although previous studies indicate that, contrary to mammals and fish, such effects are marginal in *Daphnia* and algae (Neuwoehner *et al.*, 2009; Stanley *et al.*, 2007). In the present study, the studied SSRI compounds should have been present in their more bio-available ionized form since experiments were performed in weak basic conditions (pH around 8). The used pH levels as well as high hardness water conditions are common in many semiarid and Mediterranean overpopulated rivers across Europe, America and Australia, which makes that

our results may be extrapolated to these river systems. Interestingly for 4-nonylphenol the observed responses were biphasic, enhancing reproduction at low doses and decreasing it at high doses. The two studied SSRIs only showed a monophasic response in the studied low concentration range, but previous studies indicate that they inhibited survival and reproduction at higher concentrations (Hansen *et al.*, 2008; Henry *et al.*, 2004; Stanley *et al.*, 2007). These results, thus, support the view of Calabrese (2008) that hormesis responses should be considered in ecotoxicological evaluations and are of interest for environmental toxicology, as studies investigating the long term effects of low doses of drugs are an identified research need (Fent *et al.*, 2006).

2.5. Acknowledgements

This study was funded by the Spanish Ministerio de Ciencia e Innovación (MICINN) projects CGL2008-01898 and CTM2011-30471-C02-01. MIBINN projects were co-financed by FEDER funds. Bruno Campos was supported by the Spanish grant BES-2009-022741 (FPI-MICINN).

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CHAPTER 3

Mechanisms of action of Selective Serotonin Reuptake inhibitors in *Daphnia magna*.

**Mechanisms of action of Selective Serotonin Reuptake inhibitors in
Daphnia magna.**

Bruno Campos, Benjamín Piña, Carlos Barata

Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona, 18-24,
08034 Barcelona, Spain

Published: Environmental Science and Technology, 46, (2012), 2943-2950

Abstract

Selective serotonin reuptake inhibitors (SSRIs) are known to increase offspring production in *Daphnia magna*. This study tested the hypothesis that the increase of serotonin post-synaptic activity by SSRIs changes the perception of the food environment and switches life-history responses towards higher food level: females reproduced earlier, producing more but smaller offspring. *D. magna* reproduction tests, respiration, feeding and survival-starvation assays and studies of lipids, proteins and carbohydrate levels of unexposed and exposed females to the SSRIs fluoxetine and fluvoxamine and the 5-HT serotonin receptor antagonist cyproheptadine were conducted. Factorial life-history experiments and reproductive assays showed that exposure to SSRIs increased juvenile development rate, clutch size and decrease offspring size at low and intermediate levels of food rations. These effects were reversed by the presence of cyproheptadine, indicating that 5-HT function was essential to the SSRIs effects on *Daphnia* and linking them to the pharmacological effects of SSRIs in humans. Respirometry and survival assays and biochemical analyses of lipids, proteins and carbohydrate levels showed that exposure to SSRIs increased oxygen consumption rates and decreased carbohydrate levels in adult females. These changes did not affect survival under starving conditions, but they significantly affected the capacity of the exposed animals to survive under anoxic conditions. These results suggest that SSRIs increased aerobic catabolism in *D. magna* making exposed individuals apparently more able to exploit food resources under normoxic conditions, but at the cost of being more sensitive to low oxygen levels, a common situation in natural environments.

Keywords: Selective serotonin reuptake inhibitors-SSRIs, *Daphnia*, mechanisms of action, carbohydrate metabolism, serotonin, reproduction.

3.1. Introduction

Assessing the risks of long-term exposure to low doses of pharmaceuticals is an identified research need (Fent *et al.*, 2006), particularly for those that may act as neural disruptors in non-vertebrate species (Barata *et al.*, 2004; Brooks *et al.*, 2003). For example, selective serotonin reuptake inhibitors (SSRIs) act by blocking the re-uptake of serotonin in the nerve synapses, increasing the effective concentration of serotonin in the intra-synaptic space and therefore stimulating serotonergic neurons. This effect is used worldwide to treat clinical depression in humans (Rang *et al.*, 1995), with the consequence of their widespread release into the environment. Fluoxetine levels of 12-540ng/L has been reported in US surface waters and effluents (Kolpin *et al.*, 2002), and total concentrations of SSRIs in aquatic systems were measured in the range of 840ng/L to 3.2 μ g/L (Metcalfe *et al.*, 2010; Vasskog *et al.*, 2008).

Serotonin acts through multiple receptors to mediate a great variety of functions in vertebrates and invertebrates. Different serotonin receptors, or 5-HT receptors, have been cloned in arthropods, molluscs, and nematodes (Tierney, 2001). Similarly, serotonin transporters, or SERTs, the pharmacological target of SSRIs, have been identified essentially in Metazoans (Caveney *et al.*, 2006). Whereas 5-HT receptors from arthropods (and other invertebrates) and vertebrates show structural differences that may have functional consequences (Tierney, 2001). SERTs sequences are highly conserved in different key domains (Caveney *et al.*, 2006). Not surprisingly, SSRIs designed to interact with human SERTs also exert different physiological effects in invertebrates.

SSRIs have been found to alter the reproductive physiology of *D. magna* and other invertebrates in a biphasic way (Brooks *et al.*, 2003; Flaherty and Dodson, 2005; Hansen *et al.*, 2008). Low levels of fluoxetine stimulated offspring production in *D. magna* and *Ceriodaphnia dubia* at 36 and 50 μ g/l, respectively (Brooks *et al.*, 2003; Flaherty and Dodson, 2005), but higher exposure levels inhibited reproduction in the same species (Christensen *et al.*, 2007; Hansen *et al.*, 2008; Henry *et al.*, 2004). Several SSRIs, including fluvoxamine, fluoxetine and paroxetine, induce spawning in zebra mussel (*Dreissenna polymorpha*)

within the nM and μ M range, but inhibited it at the mM range, an effect linked to the increase of serotonin activity (Fong *et al.*, 2003).

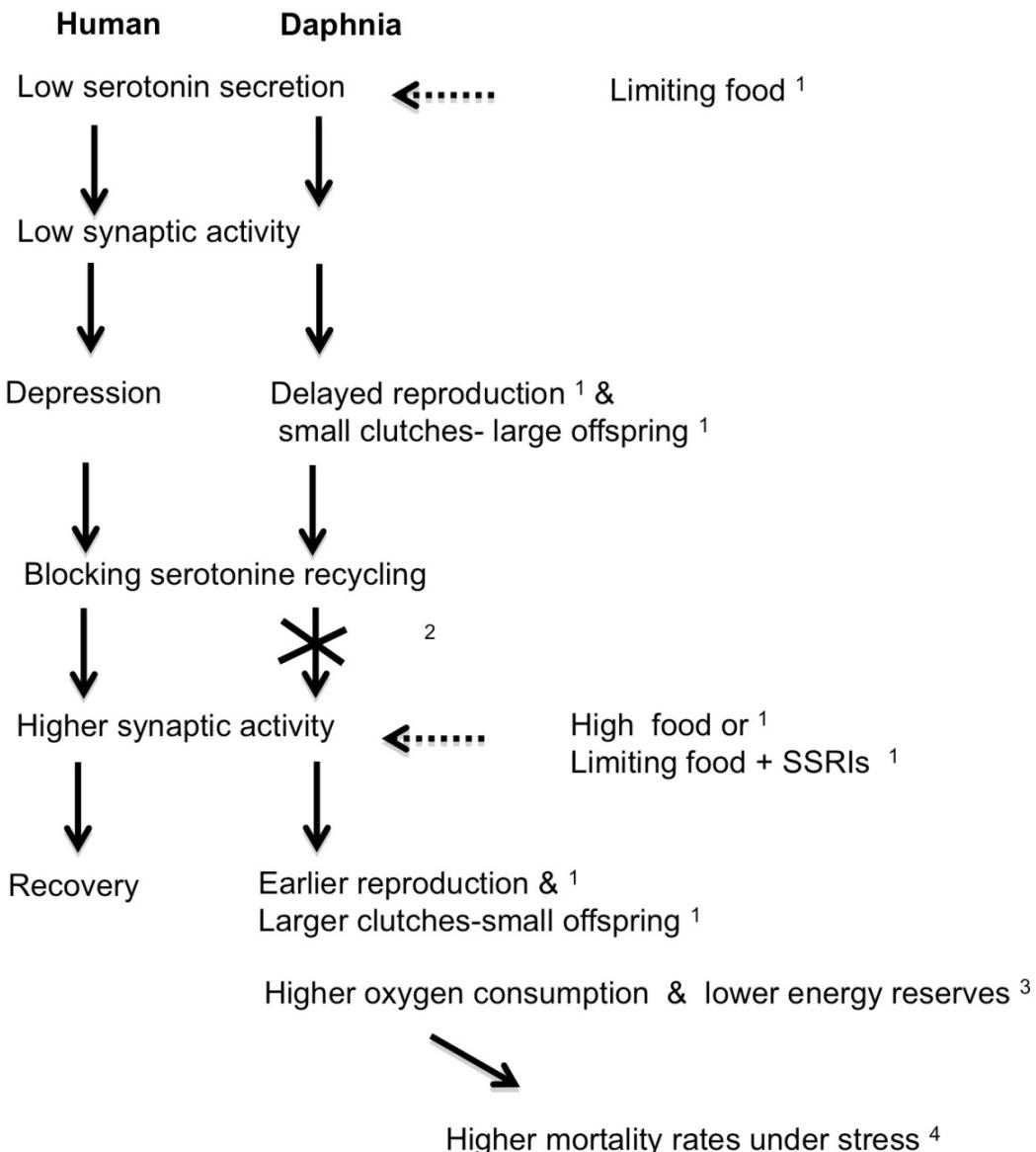


Figure 3.1. Flow chart showing the Human (left) and hypothesized *D. magna* (right) mechanism of action of SSRIs. Proposed experiments to test our four hypothesis premises are superimposed as numbers as follows. (1) Reproduction tests across food rations with fluoxetine and fluvoxamine to test the first premise that SSRIs should be able to switch *D. magna* life-history trajectories; (2) Reproduction tests with fluoxetine with the mammalian 5-HTs receptor antagonist, cyproheptadine to test that serotonin activity should be involved in these life-history changes; (3) Feeding, respiration assays and energy reserves studies to test the physiological consequences of observed life-history changes; (4) Adult survival tests under starvation with normoxia and low oxygen levels, to test for fitness costs. See the text for further information. Black arrows indicated the flow of events and dotted ones the location of *D. magna* food \pm SSRI treatments.

Serotonin may stimulate ecdysteroids and juvenile hormone in insects, which are responsible for controlling oogenesis and vitellogenesis (Nation, 2002).

Although serotonergic effects in crustacean growth and reproduction hormones is less understood, there is evidence, that monoamine neurotransmitters (serotonin, dopamine) control the secretion of peptide hormones belonging to the hyperglycaemic hormone family in malacostracean crustaceans. These hormones regulate carbohydrate metabolism, synthesis/secretion of ecdysteroids and terpenoids and hence moulting, embryo development and gonadal maturation (LeBlanc, 2007). Fluoxetine and serotonin stimulate ovarian and testicular development and increase the size of ovaries and oocytes in decapod crustaceans (Kulkarni and Fingerman, 1992; Sarojini *et al.*, 1993). Both compounds increase aggressive behaviour and carbohydrate metabolism in decapods and enhance ventilation and locomotion behaviour in amphipods (De Lange *et al.*, 2009; Huber and Delago, 1998; Kulkarni and Fingerman, 1992; McDonald *et al.*, 2011; Santos *et al.*, 2001; Sarojini *et al.*, 1993). Thus, it is possible that the observed stimulation of fecundity in *D. magna* or *C. dubia* may result from increased synaptic serotonin levels. However, since reproduction is energetically costly, such an increase in *D. magna* and *C. dubia* offspring production should not necessarily be associated with maintenance of offspring viability or fitness.

In this study we explore the hypothesis that SSRIs can affect *D. magna* juvenile developmental rates and offspring production depending on food rations and that such effects are related to the changes in serotonin activity. We tested four key aspects of this model that are summarized in Figure 3.1). In *D. magna* exposed to SSRIs under limiting food ration levels, life history trajectories will resemble conditions consistent with ample food conditions (Barata and Baird, 1998; Barata *et al.*, 2002); 2) serotonin should be involved in these life-history changes; 3) these induced changes should have a physiological impact as follows: increasing offspring production may increase metabolic rates and decrease energy reserves (carbohydrates and lipids left after reproduction); 4) observed physiological impacts should have a fitness cost, for example, compromising survival under stressful conditions such as starving or/and anoxia.

3.2. Experimental Section

3.2.1. Chemicals

Fluoxetine hydrochloride (CAS-No 56296-78-7; analytical standard, purity 100%) Sigma-Aldrich, USA), and Fluvoxamine maleate (CAS-No 61718-82-9, analytical standard, purity 100%) were purchased from Sigma-Aldrich (USA/Netherlands). All other chemicals were analytical grade and were obtained from Merck (Germany).

3.2.2. Experimental animals

All experiments were performed using a well-characterized single clone of *D. magna* (Clone F), maintained indefinitely as pure parthenogenetic cultures (Barata and Baird, 2000a). Individual or bulk cultures of 10 animals/L were maintained in ASTM hard synthetic water (ASTM, 1999) as described in Barata and Baird (2000b). Individual or bulk cultures were fed daily with *Chorella vulgaris* Beijerinck (5×10^5 cells/mL, respectively, corresponding to $1.8\mu\text{g C/mL}$; (Barata and Baird, 1998)). *C. vulgaris* was grown axenically in Jaworski/*Euglena gracilis* 1:1 medium (CCAP, 1989). Algae were harvested in the exponential phase of growth, centrifuged and then re-suspended in ASTM hard water. The number of algal cells in freshly prepared medium was checked daily from absorbance measurements at $\lambda=650\text{nm}$ in a dual-beam spectrophotometer (Uvikon 941) using standard calibration curves based on at least 20 data points, with an $r^2>0.98$. The culture medium was changed every day, and neonates were removed within 24 h. Photoperiod was set to 14h light: 10h dark cycle and temperature at $20\pm1^\circ\text{C}$.

3.2.3. Experimental design

Data from four distinct assay-experiments was considered to test the proposed mechanistic model shown in Fig 3.1:

Experiment 1: SSRIs effects on *D. magna* life-history trajectories across food rations were studied in two bioassays. Firstly, SSRIs effects on the number and size of offspring was studied in adults exposed to three concentrations of fluvoxamine (3, 7, $30\mu\text{g/L}$) and fluoxetine (10, 40, $80\mu\text{g/L}$) using a food ration level of 5×10^5 cells/mL of *C. vulgaris*. Acetone was used as a carrier solvent

(100µL/L final concentration), thus treatments also included a control and a solvent control. These experiments, hereafter referred to adult reproduction assays, started with ten 8-9 day old gravid females, which were exposed during three consecutive broods to the studied chemicals (10-14 days). Experimental adults were obtained from bulk cultures of 100 individuals reared in 10L of ASTM as described above (section 3.2.2.). Within 24h of deposition of the first clutch into the brood chamber, single females were removed and randomly assigned to each treatment. The first batch of neonates (hatching within the first 48-72h) was always discarded and not evaluated as these animals were not exposed to the tested chemicals during their entire developmental period (Barata and Baird, 2000a). Thus only neonates from the second, third and fourth broods were counted for assessing effects on total offspring production. In the third and fourth brood, body lengths of at least 10 neonates were also measured to estimate reproductive investment per offspring. Body length measurements were performed from the head to the base of the spine using a Nikon stereoscope microscope (SMZ 150, Nikon Co., Tokyo, Japan) and the ImageJ software (<http://rsb.info.nih.gov/ij/>)

In the food life-history experiment neonates (<24h old) were exposed until their fourth brood to 7 and 40µg/L of fluvoxamine and fluoxetine, respectively, at three food ration levels: 1.6×10^5 , 3.3×10^5 and 6×10^5 cells*mL⁻¹ of *C. vulgaris*, which are equivalent to 0.6, 1.2 and 2.2 1.8µg C/ml, respectively (Barata and Baird, 1998). Hereafter this food ration will be referred as low, intermediate and high, respectively, and correspond to limiting, incipient limiting and non-limiting food ration levels (Barata and Baird, 1998). Test mediums were changed daily and *C. vulgaris* was grown, harvested and counted as depicted at section 3.2.2. For each individual, age at first reproduction and total offspring production were monitored. In the third and fourth brood, the body lengths of at least 10 neonates were also considered.

Experiment 2: Role of serotonin levels in the pharmacological action of SSRIs in *D. magna*. These experiments aimed to test whether the enhancement in offspring production under SSRIs treatments could be reverted by the co-administration of a mammalian 5-HTs receptor antagonist. Gravid females were separately exposed to single and combined exposures of fluoxetine (80µg/L) and the mammalian 5-HTs receptor antagonist

cyproheptadine (10-400 μ g/L), at a food ration of 5x10⁵ cells/mL of *C. vulgaris*. The experimental set up was identical to that of adult reproduction assays and effects on total offspring production and female body length were measured.

Experiment 3. Effects of SSRIs administration on oxygen consumption, feeding rates and protein, carbohydrate and lipid levels. Gravid females were exposed to fluoxetine and fluvoxamine at 80 and 30 μ g/L, respectively, in a setup similar to the adult reproduction assays, but with 32 replicates for each treatment to achieve the critical mass required for determining protein, carbohydrate and lipid levels. Females that allocated the first brood of eggs into the brood pouch (hereafter referred as first brood females) were randomly distributed to SSRIs, control and solvent control treatments.

Feeding assays were conducted during the tests in third brood females following the same experimental protocol described for adult reproduction tests. Females were transferred into individual test vessels filled with the appropriate treatment solution plus food. Three replicate test vessels filled with the same culture medium but with no animals added were used as blank replicates. The mean initial algal cell concentration of the experimental vessels at the start of the experiment (time t0) was determined from three 5mL samples obtained from the treatment medium before it was distributed into the experimental vessels. After 24h, 5mL samples of the medium from nine vessels were selected randomly to assess the final algal cell concentration (time t24). Individual feeding rates ($\text{cells} \cdot \text{animal}^{-1} \cdot \text{h}^{-1}$) were determined as the change in cell density during 24h according to the method given by (Allen *et al.*, 1995) and converted to proportional feeding rates relative to control treatments. Algal cell density was estimated from absorbance measurements as described above.

Biochemical and Respirometry assays: Just after releasing their fourth clutch into the brood pouch, eggs were gently flushed from the brood pouch of freshly CO₂ anesthetized females following the technique of Glazier and Calow (1992). Twenty of these females were immediately frozen in liquid N₂ and stored at -80°C for biochemical determinations; whereas 12 de-brooded females were allowed to recover for 24h and then used for oxygen determination. This protocol, by using only de-brooded females excludes the contribution of developing embryos on oxygen consumption rates or on biochemical determinations (Glazier and Calow, 1992). Furthermore, the use of de-brooded

adults in the first hours of their intermolt instar ensured that the levels of lipids, carbohydrates and proteins of females were mostly somatic, as the ovaries are empty at the beginning of each instar (Glazier and Calow, 1992).

Oxygen consumption assays were performed with and without food (5×10^5 cells/mL of *C. vulgaris*). Food is known to increase oxygen consumption rates due to the metabolic costs of ingestion and digestion (Barber *et al.*, 1994). According to the mechanistic model proposed in Figure 3.1, *D. magna* exposed to SSRIs should increase swimming/locomotion activities and hence oxygen consumption rates irrespectively of food. Exposure levels of fluoxetine (80 μ g/L) and fluvoxamine (30 μ g/L) plus a control and solvent controls were tested with six replicates each. Oxygen consumption assays were performed using standard respirometry methods with 50mL gas-tight syringes (Hamilton, USA) as described in Agra *et al.* (2011), and expressed as μ g O₂ consumed per organism per hour. Six syringes were used per treatment and food levels. They were filled with 30mL of the appropriate test solution and with one fourth brood adult. The remaining air was expelled from each syringe, which was left in the dark in a water bath (20°C) for 24 h. After the first 2h of exposure, initial O₂ concentrations were measured with the aid of an oxygen meter (Model 782 Strathkelvin Instruments, Glasgow) provided with an oxygen electrode (model 1302, Strathkelvin Instruments, Glasgow) and after 24h from the start of the test, the final O₂ concentrations were measured in the same way. The oxygen consumption was given by the differences in the oxygen content of water before (T₀=2 h) and after (T_{final}=24 h) the exposure period. Respiration rate was expressed as ug O₂ consumed per organism per hour. Within each experiment three blank controls (syringes with no organisms) were tested for every treatment to correct for the ambient O₂ depletion due to factors other than organism's respiration. The depletion in the oxygen content on these blank controls was used as a correction factor for the appropriate treatments.

Total lipids, carbohydrates, and proteins were extracted as described in Cheng *et al.* (2011 and De Coen and Janssen (1997). Pools of 5 adults were used to achieve sample requirements with a total of 4 replicates per treatment. Groups of 5 adult females were homogenized in 800 μ L ice-cold 100mM phosphate buffer (PBS), pH 7.4. After homogenization, 500 and 200 μ L of homogenate were used for lipid contend and carbohydrate and protein

determination, respectively. Lipids were extracted with chloroform: methanol 1: 1. After centrifugation at 10,000g the top phase was removed and the solvent evaporated at 90°C. After addition of concentrated H₂SO₄, the mixture was heated for 20 min at 90°C. 50µL of vanillin-phosphoric acid was added and left to develop colour for 10 minutes. The total lipid content expressed as µg per individual was determined at 540nm using trypalmitin as standard (Cheng *et al.*, 2011). To determine total protein and carbohydrate (sugar) content, 15% trichloroacetic acid (TCA) was added to homogenates and samples were incubated at -20°C for 10 min. After centrifugation at 10,000g, the pellet formed was washed with 5% TCA and both supernatant fractions were combined and used for the total sugar analysis. The remaining pellet was re-suspended in NaOH and incubated at 60°C for 30 min and then neutralized with HCl. Total protein content was determined using the Bradford's method (Bradford, 1976) and bovine serum albumin as standard. Total carbohydrate content of the supernatant fraction was quantified by adding 5% phenol and H₂SO₄ to the extract. After 30min incubation at 20°C, the absorbance was measured using glucose as a standard at 492nm. The protein and carbohydrate content was expressed as µg per animal. All measurements were performed with the Multi-Detection Micro- plate Reader, BioTek® (Vermont, USA).

Experiment 4: Effects of SSRIs on adult survival under starving conditions. Two separate experiments were performed to tests survival responses under normoxia and low oxygen levels, respectively. In the first bioassay first brood females (20 replicates per treatment) were randomly distributed to SSRIs (80µg/L fluoxetine and 30µg/L fluvoxamine), control and solvent control treatments. Just after releasing their fourth brood into the brood pouch ten females of each treatment were randomly assigned to control or treatment medium without food. Survival of exposed organisms was monitored at 12h intervals until all individuals died.

The experimental set up for the second bioassay was identical to the previous one but females were starved to the same treatment medium under low oxygen levels (1mg/L). Low oxygen levels were obtained by bubbling N₂ into the test medium and maintained animals in 100mL sealed glass bottles until death. Survival of exposed and control organisms were monitored at 8h intervals until all individuals died.

3.2.4. Physical-chemical analyses

Duplicated water samples of freshly prepared and old (24 h) test solutions were collected at the beginning and end of the tests to determine that measured oxygen levels and pH were within the limit established by OECD guidelines (OECD, 1981). Dissolved oxygen concentration (DO) was measured using an oxygen electrode model 1302 (Strathkelvin Instruments, Glasgow) and pH was measured using an epoxy-body combination electrode, coupled to a Crison micro pH 2001 meter and calibrated with standard pH buffer solutions (Sigma, Madrid, Spain). Mean oxygen levels were 95 ± 3.4 % of saturation and pH values 7.3 ± 0.2 for all experiments, except for those conducted under anoxia, in which mean initial and final oxygen levels were 1.4 ± 0.2 and 0.4 ± 0.04 mg O₂/L, respectively.

Stability of the compounds during the test was confirmed using solid-phase extraction and liquid chromatography-tandem mass spectrometry (LC-MS-MS). Analysis of fluoxetine 10,40, 80µg/L and fluvoxamine 3,7, 30µg/L was restricted to final test solutions (24h) in experiments 1-3 and to initial ones in experiments 4-5 and conducted with the method of Gómez *et al.*, (2006). For the pre-concentration of fluoxetine and fluvoxamine, Oasis 60mg SPE cartridges were conditioned with 10mL of methanol followed by 10mL of water. 100mL of water were pre-concentrated at a flow rate of 10ml/min and eluted with 2x4mL of methanol. This extract was then reduced under nitrogen to incipient dryness and reconstituted in 1mL with methanol. Solid phase extraction recoveries were determined using appropriate amounts of analytes spiked into water before extraction and in 1ml of the extracts after extraction.

The LC analysis were performed using a Waters 2690 HPLC separations module (Milford, MA, USA) equipped with a Purospher Star RP-18 end-capped column (125mm×2.0mm, particle size 5µm) and C18 guard cartridge supplied by Merck (Darmstadt, Germany). The following gradient was chosen for HPLC separation at room temperature: eluent A was acetonitrile and eluent B was formic acid 0.1% in MilliQ water at a flow rate of 0.2mL/min. The elution started with 10% of eluent A, followed by a 25-min gradient to 80% of eluent A and a 3-min gradient to 100% of eluent A and then back to the initial conditions within 4 min. An injection volume of 10µL was used. The tandem MS analyses were carried out on a Micromass Quattro triple quadrupole mass spectrometer

equipped with a Z-spray electrospray interface (Manchester, UK). Instrument control, data acquisition and evaluation were done with the Masslynx NT software (v.3.4). The parameters for the analysis were: electrospray source block and desolvation temperature: 150 and 350°C, respectively; capillary and cone voltages: 2.8kV and 30V, respectively; argon collision gas 2.5×10^{-3} mbar; cone nitrogen gas flow and desolvation gas: 43 and 628L/h. Mass spectrometer was operated in the multiple reaction monitoring MRM mode with unit mass resolution on both mass analyser.

Identification criterion of the target compounds was based on: (a) LC retention time of the analyte compared to that of a standard ($\pm 2\%$) and (b) the ratio of abundances of two specific precursor ion to product ion transitions (within 10% of the ratios obtained for the standard). Mass to charge ratios used for MS quantification and identification were m/z 310 to 44 and 148 for fluoxetine and m/z 319 to 71 and 200 for fluvoxamine. Quantification was based on external calibration standard 8 point curves ($r^2 > 0.98$). Limits of detection and quantification defined as the minimum detectable amount of analyte with a signal to noise ratio of 3:1 and 10:1, respectively, were determined from the spiked water samples.

Mean retention time was 12.91 and 19.90 min for fluvoxamine and fluoxetine, respectively.

3.2.5. Data analyses

The effect of food rations and treatment or of treatment on measured life history and physiological responses were analysed by two and one way ANOVA. When significant ($P < 0.05$) treatments effects occurred, post-hoc Dunnet's tests were performed to compare exposure treatments with solvent controls. Prior to analyses all data except survival responses was log transformed to achieve normality and variance homoscedasticity (Zar, 1996). Survival responses were assessed by Wilcoxon-Gehan tests. Tests were performed with the IBM SPSS Statistics software version 19.

3.3. Results and Discussion

3.3.1. Contaminant levels in water

In all tests performed measured (Mean \pm SD, $n=4-10$) SSRIs concentrations were close to nominal levels being for fluvoxamine 2.1 ± 0.3 , 7.5 ± 0.4 , $26.5\pm3.4\mu\text{g/L}$ and for fluoxetine 8.7 ± 0.5 , 45.3 ± 3.6 , $76.1\pm4.9\mu\text{g/L}$, respectively. For the sake of clarity, results are referred to nominal values.

3.3.2 Life-history responses

Following exposure of adult females to fluvoxamine or fluoxetine the total number of offspring increased (ANOVA, $F_{7, 57} = 4.5$, $P<0.05$) when compared to controls (Figure 3.2), with no significant changes in the offspring average size (ANOVA, $F_{7, 116} = 0.9$, $P>0.05$). These results are in accordance with previous reports of increased offspring production by fluoxetine at concentrations similar to the ones used in this study (Flaherty and Dodson, 2005). Therefore, we conclude that this effect is a consistent consequence of the presence of SSRIs.

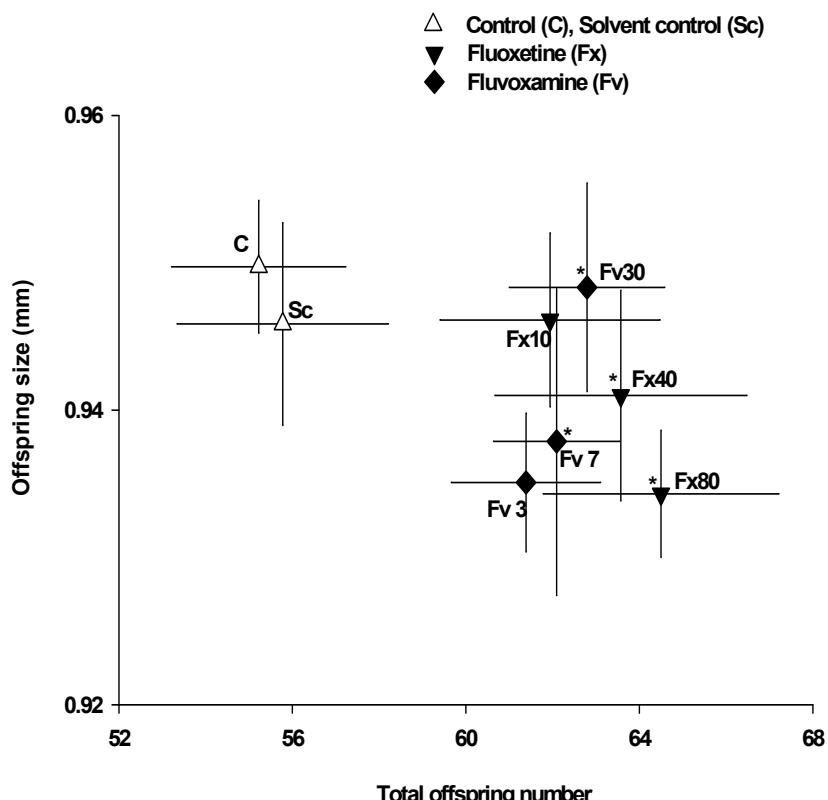


Figure 3.2. Effects of fluoxetine (10, 40, 80 $\mu\text{g/L}$) and fluvoxamine (3, 7, 30 $\mu\text{g/L}$) on offspring size and number in exposed adult females. Values are Mean \pm SE; N=10. Numbers after Fx and Fv indicate exposure levels. * Total offspring significant ($P<0.05$) differences from solvent control treatments following ANOVA and Dunnet's post hoc tests.

Reducing the amount of food modulated the effects of SSRIs exposure on offspring size and number in *D. magna*. SSRIs effects were abolished at high-food conditions (black symbols in Figure 3.3A), whereas at intermediate and low food rations (grey and white symbols in Figure 3.3A), SSRIs exposure effects on offspring number and size become more evident (Figure 3.3A, see ANOVA results in Table 3.1). In addition, females exposed to fluvoxamine reproduced earlier than control ones only at low and intermediate food rations (white and grey bars, Figure 3.3B, see also ANOVA results in Table 3.1). These results are consistent with the hypothesis that *D. magna* individuals exposed to SSRIs modified their perception of food ration levels switching life-history responses towards higher food rations, and that such changes were closely related to the pharmacological mode of action of SSRIs of increasing serotonin postsynaptic activity, and resulting in an increased offspring production and decreased offspring size and maturation age (see the experimental hypotheses depicted in Fig. 3.1, (Barata and Baird, 1998)).

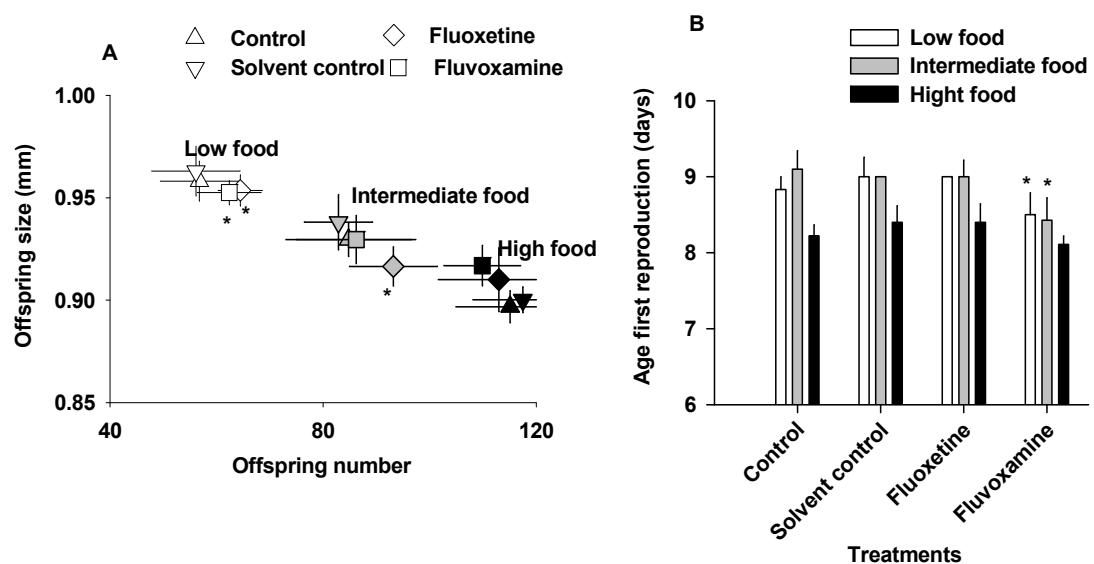


Figure 3.3. Effects of fluoxetine (40 $\mu\text{g/L}$) and fluvoxamine (7 $\mu\text{g/L}$) on offspring size and number (A) and age at first reproduction (B) on *D. magna* individuals reared across food rations (1.6×10^5 , 3.3×10^5 , 6×10^5 cells mL^{-1} of *C. vulgaris*, respectively). Values are Mean \pm SE, N=9-10; * significant ($P<0.05$) differences from solvent control treatments following ANOVA and Dunnet's post hoc tests

The pharmacological action of SSRIs inducing offspring production by increasing postsynaptic serotonin activities in *D. magna* was investigated by blocking serotonergic activity with the mammalian 5-HTs receptor antagonist, cyproheptadine. Cyproheptadine alone significantly inhibited total offspring production in a concentration related manner above 50 $\mu\text{g/L}$ (ANOVA,

$F_{5,50}=17.6$; $P<0.05$, Figure 3.4A). Exposures of adult females to 80 μ g/L of fluoxetine produced significantly (ANOVA, $F_{6,60}=5.0$; $P<0.05$) more offspring than those in solvent controls but not when they were co-exposed to the same concentration of fluoxetine and 10 or 50 μ g/L cyproheptadine (Figure 3.4 B). Cyproheptadine by itself at 10 or 50 μ g/L did not affect reproduction, as females exposed to this antagonist alone had similar fecundity as those of solvent controls (Figure 3.4 B). These results support the hypothesis that SSRIs like fluoxetine act on *D. magna* reproduction increasing post-synaptic serotonin activity in its receptor 5-HT, a mechanism similar to its intended pharmacological effect in mammals.

Table 3.1. ANOVA results testing the effects of food ration levels and treatment on selected life history responses of *D. magna* individuals exposed to the studied SSRI at three food regimes. Offspring size analyses were performed on third and fourth clutch neonates. Only degrees of freedom (df), Fisher's coefficient (F) and probability values (P) are depicted.

Trait	Factor	df	F	P
Age ¹	Food	2	17.1	<0.01
	Treatment	3	12.7	<0.01
	Treatment x Food	11	33.1	<0.01
Total offspring ²	Food	2,94	365.6	0.00
	Treatment	3,94	3.1	0.03
	Treatment x Food	6, 94	2.3	0.04
Offspring size ³	Culch	1,158	101.7	<0.01
	Food	2,158	8.5	0.00
	Treatment	3,158	2.8	0.04
Body length ²	Treatment x Food	6, 158	2.8	0.01
	Food	2,98	293.7	0.00
	Treatment	3,98	0.2	0.88
	Treatment x Food	6, 98	1.0	0.43

¹ Age at first reproduction was analysed by non-parametric Kruskal-Wallis tests; ² effects on total offspring production and body length were tested by two way ANOVA; ³ Offspring size versus offspring number was analysed using ANCOVA analyses considering offspring number as a covariate.

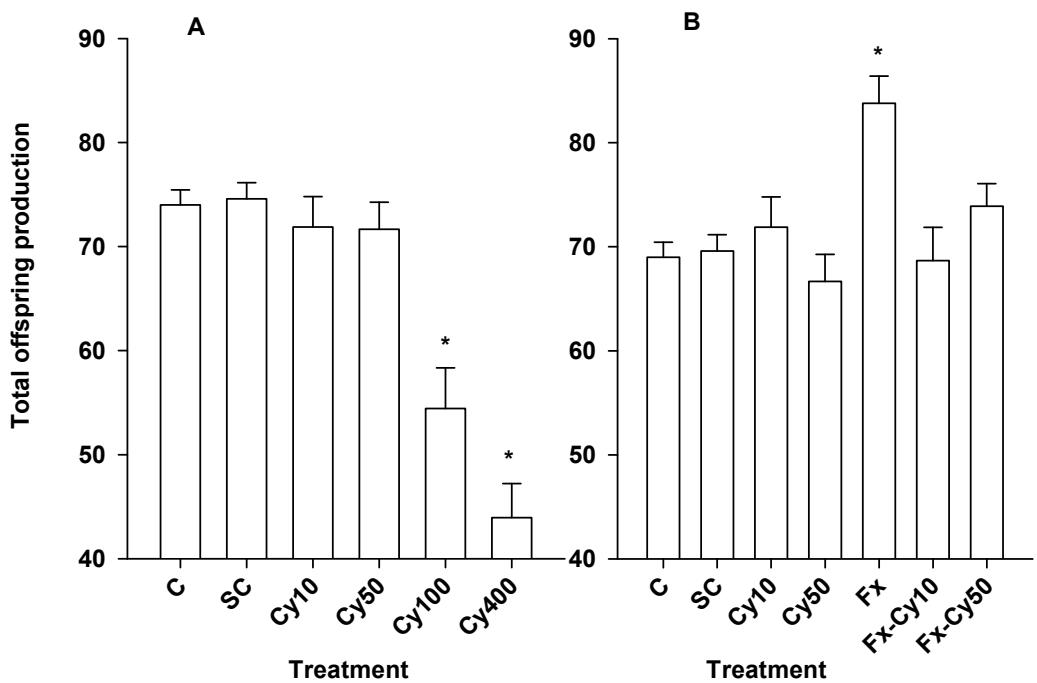


Figure 3.4. Effects of Cyproheptadine (cy) alone (A) and of cyproheptadine (10, 50 μ g/L) co-administered with fluoxetine (Fx, 80 μ g/L (B) on offspring number. Values are mean \pm SE, N=10, * significant ($P<0.05$) differences from solvent control treatments following ANOVA and Dunnet's post-Hoc tests.

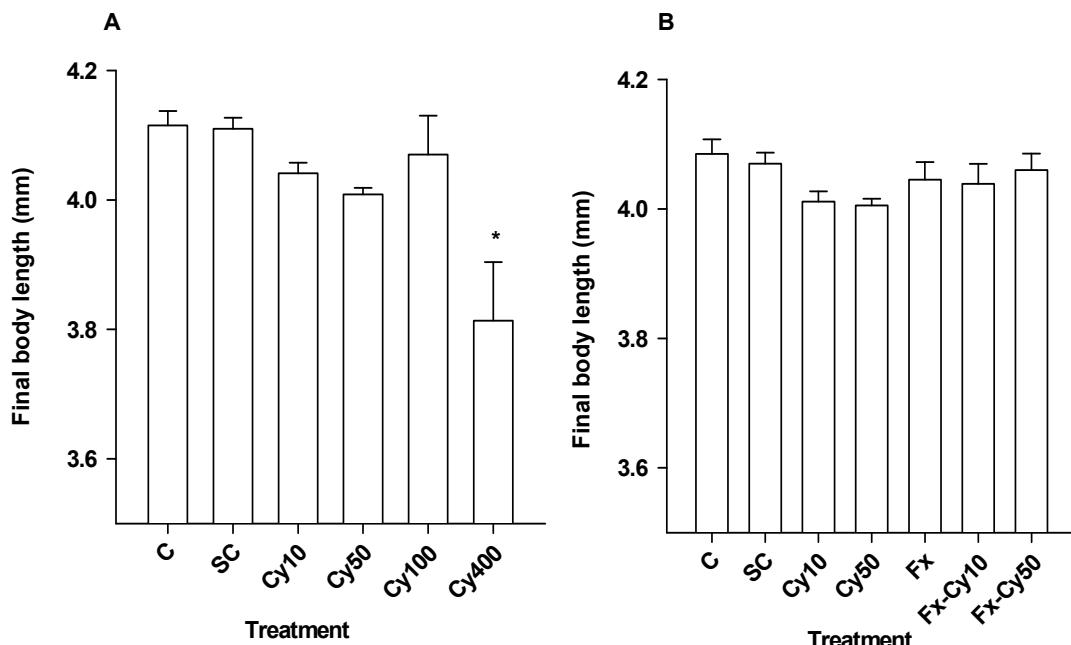


Figure 3.5. Effects of cyproheptadine (cy) alone and of cyproheptadine (10, 50 μ g/L) co-administered with fluoxetine (Fx, 80 μ g/L) on body length. Values are Mean \pm SE, N=10; * significant ($P<0.05$) differences from solvent control treatments following ANOVA and Dunnet's post hoc tests.

Cyproheptadine has previously been shown to prevent serotonin from inducing spawning in bivalves (Fong *et al.*, 2003) and ovary growth and oocyte maturation in the prawn *Macrobrachium rosenbergii* (Meeratana *et al.*, 2006). Furthermore, our results also show that cyproheptadine acts as a true 5-HT antagonist because at high exposure levels (100 µg/L) it specifically inhibited reproduction (Figure 3.4A) without affecting growth (see also body length effects in Figure 3.5). The results of the present blocking experiments, thus, support the assumption that SSRIs are, in fact, blocking reuptake, increasing the availability of synaptic serotonin, and activating post-synaptic receptors. Thus, it is the release of serotonin that is inducing offspring production and therefore being blocked by cyproheptadine. Although synaptic serotonin levels or post-synaptic serotonin activity were not measured in the present study, there is evidence that serotonin as well as 11 other biogenic amines and their metabolites are present in *D. magna* tissues and their concentration are modulated during the daily cycle (Ehrenstrom and Berglind, 1988).

3.3.3. Physiological effects

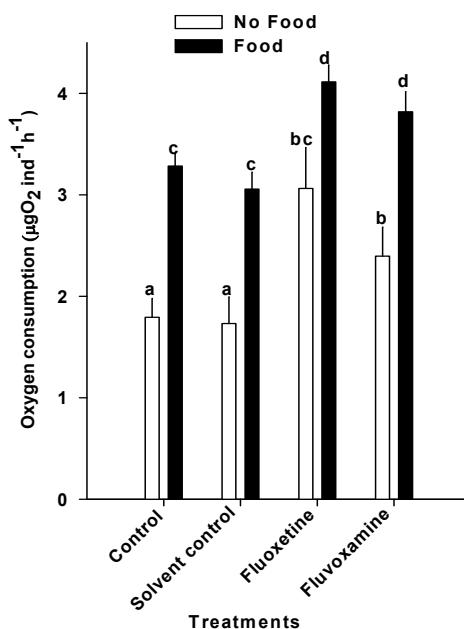


Figure 3.6. Effects of fluoxetine (80µg/L) and fluvoxamine (30µg/L) on adult oxygen consumption rates. White and black bars correspond to responses without and with food. Values are Mean±SE: N=6. Different letters indicate significant ($P<0.05$) differences among treatment groups following ANOVA and Tukey's post hoc tests.

Fed *D. magna* had significantly increased oxygen consumption rates compared with starved individuals ($P<0.05$; ANOVA; food effect $F_{1,39} = 51.5$; Figure 3.6), a

well-known characteristic of *Daphnia* physiology (Philippova and Postnov, 1988).

D. magna responded to SSRIs exposure by increasing oxygen consumption rates ($P<0.05$; SSRIs effect $F_{3,39} = 7.8$), but SSRIs did so independently of food ration (not significant, $P<0.05$, interaction term; $F_{3,39} = 0.9$). *D. magna* individuals exposed to SSRIs did not have their feeding rates significantly affected (ANOVA, $F_{3,30} = 1.6$; Figure 3.7).

Whereas in absence of food, oxygen consumption rates in *Daphnia* provide good estimates of maintenance costs that include basal metabolism and swimming/ ventilation activities that are necessary to live, food ingestion is energetically expensive accounting for almost 40% of metabolic or respiration rates (Barber *et al.*, 1994; Philippova and Postnov, 1988). Pollutants may increase maintenance costs by increasing detoxification mechanisms (demand side effects), but more often, when food is present, pollutants decrease metabolic rates inhibiting food intake (supply side effects) (Barata *et al.*, 2004; Barber *et al.*, 1994).

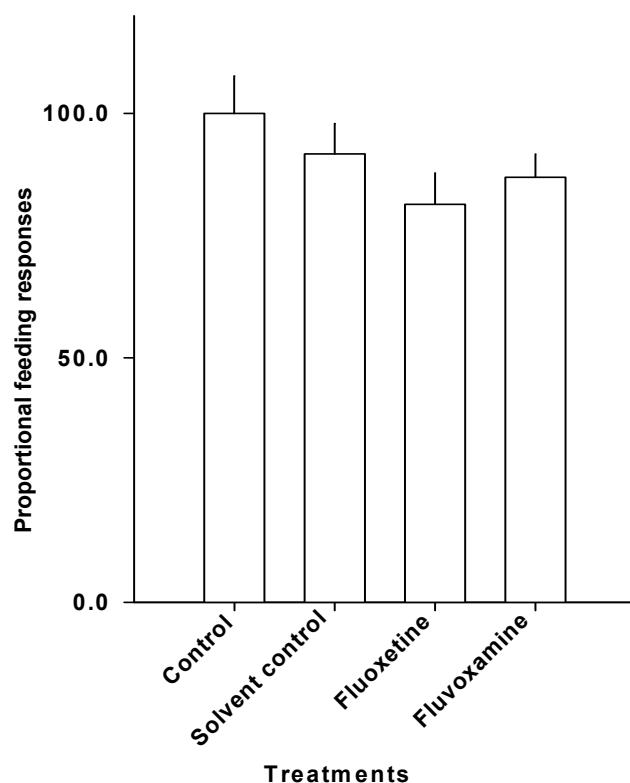


Figure 3.7. Effects of fluoxetine (80 μg /L) and fluvoxamine (30 μg /L) on proportional feeding rates relative to control treatments. Values are Mean \pm SE; N=10.

Our results suggest that SSRIs increase maintenance costs, either by increasing swimming/ventilation activity, detoxification and/or other physiological processes. One of such physiological processes could be the metabolic cost of having higher reproductive investment. We consider, however, that this factor cannot explain by itself the observed effects. Considering an averaged egg mass of 5 µg d.w. and an egg mass oxygen consumption rate of 2.7µg O₂*mg⁻¹d.w.*h⁻¹ (Glazier and Calow, 1992), the estimated increases of oxygen consumption rates associated to observed increases of fecundity of 5-10 eggs per brood under SSRIs treatments were too low (0.13µg O₂*h⁻¹) to account for the observed 0.7-1.3µg O₂*h⁻¹ increase on oxygen consumption (Figure 3.6). Therefore, other physiological processes, like increasing swimming/ventilation activity and enhanced levels of catabolism (i.e. carbohydrate metabolism) should contribute significantly on the observed 20-50% increase in oxygen consumption rates (De Lange *et al.*, 2009; Huber and Delago, 1998; Kulkarni and Fingerman, 1992; McDonald *et al.*, 2011; Santos *et al.*, 2001; Sarojini *et al.*, 1993). Analyses of putative energy reserves in de-brooded *D. magna* females showed that SSRIs significantly decreased ($P<0.05$) carbohydrate levels (ANOVA, $F_{3,12} = 11.0$) but not those of lipids ($F_{3,12} = 3.2$) and proteins ($F_{3,12} = 2.9$, Figure 3.8).

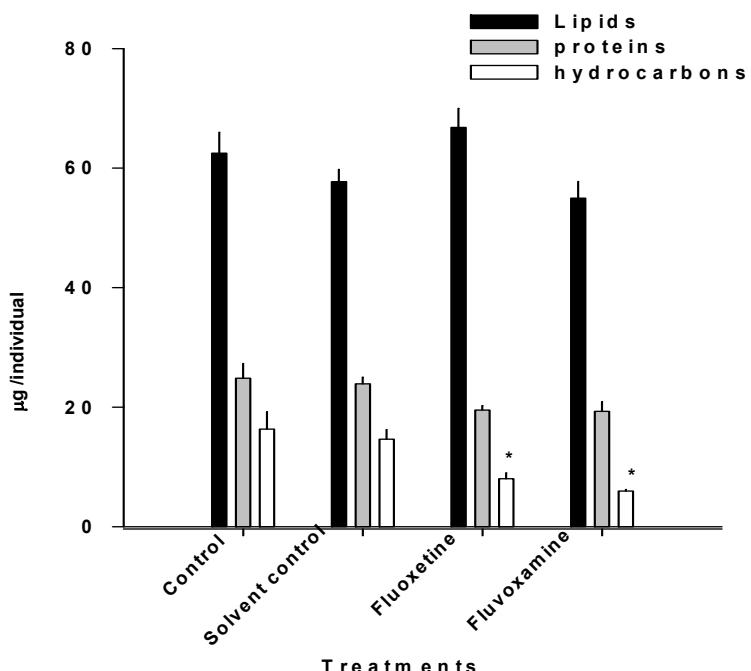


Figure 3.8. Effects of fluoxetine (80µg/L) and fluvoxamine (30µg/L) on lipid, carbohydrate and protein levels of exposed adults. Values are Mean±SE. N=4; * significant ($P<0.05$) differences from solvent control treatments following ANOVA and Dunnet's post hoc tests.

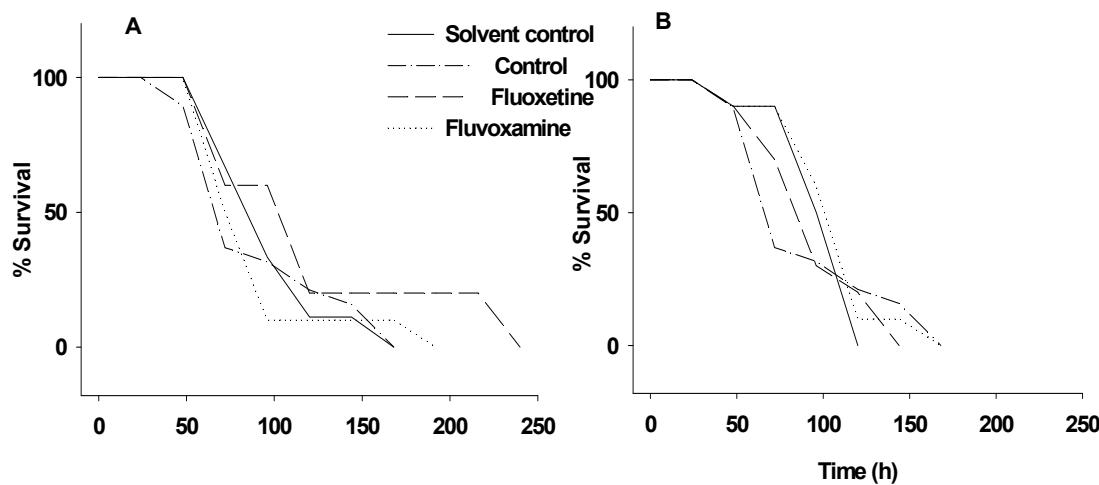


Figure 3.9. Effects of fluoxetine (80 μ g/L) and fluvoxamine (30 μ g/L) on survival responses of adults under starvation. For clarity, longevity responses of adults pre-exposed to the studied SSRIs and starved in clean water (A) or in water plus SSRI (B) are separated.

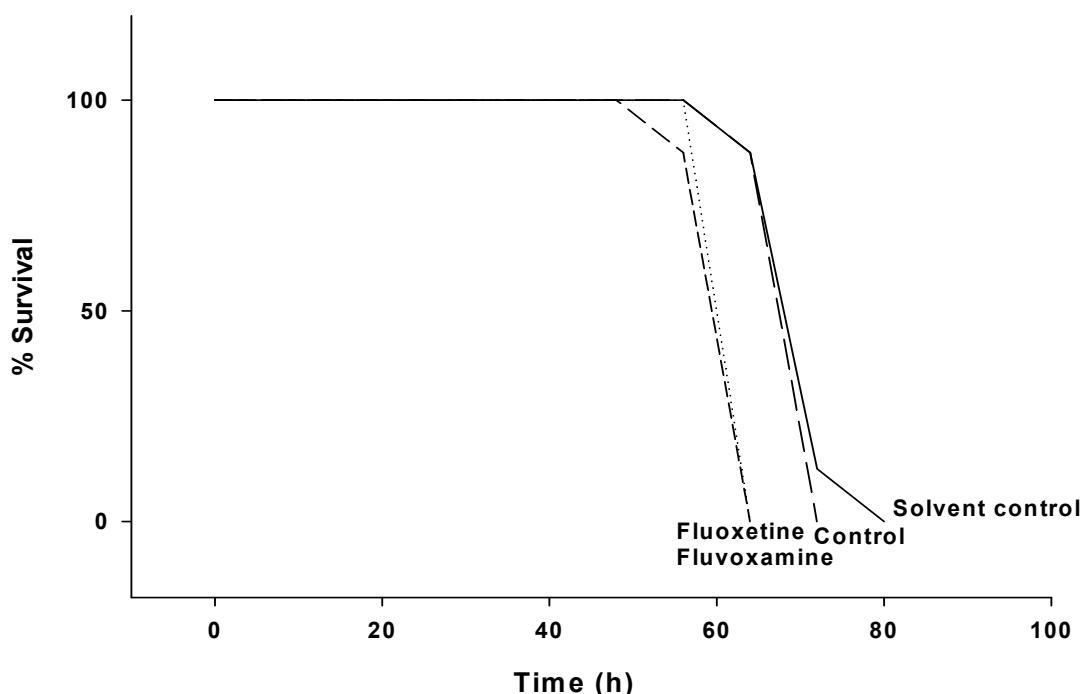


Figure 3.10. Effects of fluoxetine (80 μ g/L) and fluvoxamine (30 μ g/L) on survival responses of adults under starvation and anoxia.

Injection of serotonin and fluoxetine in several species of decapods increased glucose and hyperglycaemic hormonal levels in haemolymph (Santos *et al.*, 2001). As crustacean hyperglycaemic hormones are present in central and peripheral nervous systems of *D. magna* (Zhang *et al.*, 1997), these observations may be fully applicable to our results. A suitable model would be that SSRIs, by increasing synaptic serotonin levels, up-regulate hyperglycaemic hormones and hence increasing carbohydrate metabolism.

Finally, survival responses under starving conditions of *D. magna* females pre-exposed to SSRIs was determined in water only and in water spiked with the studied SSRIs exposure levels under normoxic and low oxygen levels (1mg/L). Under normoxic conditions survival tests did not evidence significant ($P<0.05$) differences among treatments (Wilcoxon-Gehan tests=5.4; df=6; Figure 3.9). This means that the observed increasing oxygen consumption rates and depleted carbohydrate reserves did not have measurable adult fitness costs in terms of tolerance to starving conditions. These unexpected results apparently contradict life-history theory that should predict lower survival of individuals having similar energy reserves but greater 20-50% oxygen consumption rates (Sibly and Calow, 1986). Indeed, using the energetic equivalent values reported for oxygen consumption (484kJ/mol O₂), lipid (39500mJ/mg), protein (24000mJ/mg) and carbohydrates (17500mJ/mg) (De Coen and Janssen, 1997), and considering the data reported in Figures 3.6 and 3.8, it was estimated that *D. magna* females starved and exposed to SSRIs should exhaust all their energetic reserves in 70 hours, whereas those of controls should last for 120 hours. Nevertheless, these predictions assume a constant oxygen consumption rate during starvation, and that oxygen consumption rates are directly related to aerobic metabolism in both SSRIs and control daphnids. Both assumptions may not be exactly correct, as *D. magna* can decrease its oxygen consumption rates during starvation (Glazier and Calow, 1992) and it may modulate aerobic and anaerobic metabolism to adapt to high and low oxygen conditions (Paul *et al.*, 1998). A plausible hypothesis is that SSRIs may increase oxygen consumption rates by favouring aerobic metabolism at the expenses of the anaerobic one. As aerobic metabolism is energetically more efficient, daphnids exposed to SSRIs under starving conditions may use energy more efficiently than unexposed individuals, without

compromising survival. However, animals with higher oxygen consumption rate and low carbohydrate reserves should be more sensitive to anoxia, due to their dependence on aerobic metabolism.

Figure 3.10 shows that exposure to SSRIs significantly reduced survival under low oxygen conditions (1 mg/L, Wilcoxon-Gehan tests = 22.7; df = 3; P<0.05). These results support the argument that SSRIs enhance aerobic metabolism, making the exposed individuals more susceptible to anoxia. In real field situations *D. magna* individuals often encounter low oxygen conditions and are adapted to live for certain periods under anoxia (Paul *et al.*, 1998; Zeis *et al.*, 2009). This means that in particular situations of low oxygen levels observed life-history and physiological effects of SSRIs on *D. magna* individuals may bear a fitness costs in terms of increasing mortality rates.

D.	pulex	6	NLSGKVSGLAAAADEQHEPILLNHPDDQQDEDKAQIVFFLSETTAVSAGSH-RPDAPND N + +S D Q + L P + QI S + AG R P	64
Human		7	NSQKQLSACEDGEDCQENGVLQKVVPTEGDKVESQQISNGYSAVSPSPGAGDDTRHSIPAT	66
D.	pulex	65	SPAL-----EERETWAKKAEFLALAVIGFAVDLGNVWRFPYICYKNGGGAFLIPYVVMV + L ERETW KK + FLL+VIG+AVD LGNVWRFPYICY+NGGG AFL+PY + M +	118
Human		67	TTTLVAELHQGERETWGKKVDFLLSVIGYAVD LGNVWRFPYICYQNGGGAFLLPYTIMAI	126
D.	pulex	119	FGGLPLFLFYME LALGQFHRSGCLTLWKR KICPA LKG VGYAICI IDF YMGM YYNTI I GWA VYY FGG+PLFLFYME LALGQ+HR+GC++W++ICP KG+GYAICII FY+ YYNTI+ WA+YY	178
Human		127	FGGIPLFLFYME LALGQYH RNC CISI WRK ICPI FK GIG YAICI IA FYIAS YYNTI MA W ALYY	186
D.	pulex	179	FVASFTSEL PWTSCDHPWNTN S CALVG PVDNGTFV ---RSPAQEYFERNVLEN YRS DGM D ++SFT +LPWTSC + WNT +C DN T+ SPA+E++ R+VL+ +RS G+	235
Human		187	LISSFTDQLPWTSCCKNSWNTGNCTNYFSEDNITWT LHST SPAEEFYTRHVLQI HRS KGLQ	246
D.	pulex	236	DLGPIKWSLALCVFAVFV L VYFSLWKGVRSTGKAVWITA VAPYIVL I ILLFRGASLPGAG DLG I W LALC+ +F ++YFS+WKG V++GK VV+TA PYI+L +LL RGA+LPG A	295
Human		247	DLGGISWQLALCIMLIFTVIYFSIWKGVKTSGKVVWVTATFPYI ILSVLLVRGATLPGAW	306
D.	pulex	296	DGIRYFLTPQWSKLAETKVW TDAA SQVFFSLGP GFGT LLALSSYNKFHN NCYFD ALLTSS G+ ++L P W KL ET VW DAA+Q+FFSLGP GFG LLA +SYNKF+NNC+ DAL+TS	355
Human		307	RGVLFYLKPNWQKLLETGVWIDAAA QIFFSLGP GFGVLLAFAS YNKFNN NCYQ DALV TS V	366
D.	pulex	356	INLAT SLLAGF VIFAVLG YMAEIRN VSID QL GLE-GP GLV FV VYPE AIA TMAG STFW SMI +N TS ++GF VIF VL GYMAE+RN + ++ + GP L+F+ Y EAIA M STF++ +I	414
Human		367	VNCMTSFVSGF VIFTVLGYMAE MRNEDVSEVAKDAGPSLLFITYAE AIA NM PAST FF AII	426
D.	pulex	415	FFFLLITLGLDSTFGGLEAMITGLCDEYPVLLGRRREL FVGILLVFIYL CALPTTTYGGM FF +L ITLGLDSTF GLE +IT + DE+P + +RRE FV +++ + +L T T+GG	474
Human		427	FFLMLITLGLDSTFAGLEG VITAVLDEFPHVWAKR RERFV LAVVITCF GSVL TLT FGGA	486
D.	pulex	475	YLV D L L N VYGP GIA I I LFL V F V B AMG V SW CYGT QRF SDDIESMLG F Q PGP FW KIT WAY VSP Y+V LL Y G A+L + +EA+ VSW YG +F D++ MLGF PG FW+I W +SP	534
Human		487	Y VVK L L E E Y ATGP AVL TVALIEA VAVS WF Y GIT QFC RDV KEM LGF SP GFW RIC WVA I SP	546
D.	pulex	535	I FILLI FIC TLIDPVPLDT QDY TYP AW SIKVG WLTA I PL SC I PI YMI YK LI I TKG TF VQ +F+L I L+ P L Y YP WSI +G+ + CIP Y+ Y+LI I T GTF +	594
Human		547	LFL L L F I C SFL M SPP Q L R L F Q YN Y P Y W S I I L G Y C I G T S S F I C I P T Y I A Y R L I I T P G T F K E	606
D.	pulex	595	RLVKMFTPEDTSD 607 R+K TPE ++	
Human		607	RIIKSITPETPT E 619	

Figure 3.11. Alignment of a putative *Daphnia pulex* SERT (DAPPUDRAFT_314292) and its closest human relative (ref|NP_001036.1, sodium-dependent serotonin transporter). Identical residues (51%) are represented in the central row, "+" signs correspond to conservative amino acid changes (69% of total homology). Numbers correspond to the amino acid position in both sequences. Analysis performed using the BLAST server at <http://www.ncbi.nlm.nih.gov/blast>

Our working hypothesis implies that SSRIs acts in *Daphnia* following a mechanism of action similar or identical to their intended pharmacological effect in humans, blocking serotonin reuptake and increasing serotonin post-synaptic activity (Wong *et al.*, 1995). No *bona fide* SERT has not been characterized in *Daphnia* yet. However, an apparently complete sequence from the *Daphnia pulex* genome (DAPPUDRAFT_314292) encompasses a putative protein with 51% identity and 69% homology to its closest human relative, a Na-dependent SERT (Figure 3.11).

Whether or not this putative sequence corresponds to a functional SERT is unknown but its presence suggests that *Daphnia* genome codifies SERT proteins structurally similar to human SERTs, constituting therefore putative targets for SSRIs. Nevertheless, the physiological effect of SSRIs in human and *Daphnia* appears to differ. In humans, SSRIs re-establish serotonin levels and ameliorate depression and other neuronal disorders (Wong *et al.*, 1995). In *D. magna*, they change the perception of food availability, switching life-history responses at limiting food rations. Under these conditions females exposed to SSRIs produced more but smaller offspring and matured earlier than non-exposed ones. Unexpectedly, no detrimental side effects of SSRIs on *D. magna* females were observed in aerobic conditions, as they showed similar survival rates under starving conditions than unexposed individuals, despite of their greater oxygen consumption rates and less carbohydrate reserves. Nevertheless, under anoxia conditions SSRIs exposed organisms died earlier than unexposed ones. These results indicate that SSRIs could increase aerobic catabolism in exposed females making them more efficient using their energetic reserves, yet less able to sustain aerobic respiration over time. *D. magna*, like many other species, modulates its aerobic and anaerobic catabolism to maximize its survival across anoxia and other stressful environments (Paul *et al.*, 1998; Zeis *et al.*, 2009). If SSRIs increases neuronal activity and carbohydrate metabolism in *D. magna* they may also increase aerobic metabolism making exposed organisms energetically more efficient but at expenses of consuming more oxygen.

Being less tolerant to low oxygen levels may be ecologically and evolutionary critical in zoo-planktonic species like *Daphnia* that in lakes have a diel vertical migration to avoid fish predation, eating in shallow waters at night and migrating

to deep waters that usually have less oxygen during daylight (Cousyn *et al.*, 2001). Nevertheless, further research with more neurological disrupters and doses should be performed to confirm the effects reported in this study.

3.4. Acknowledgments

This work was supported by the Spanish MICINN grants (CGL2008-01898/BOS and CTM2011-30471-C02-01) and FEDER funds. Bruno Campos was supported by a fellowship from the MICINN (FPI BES-2009-022741). Chemical analyses were performed with the aid of the Mass Spectrometry Service from the Department of Environmental Chemistry of IDAEA (CSIC).

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CHAPTER 4

Identification of metabolic pathways in *Daphnia magna* explaining hormetic effects of Selective Serotonin Reuptake Inhibitors and 4-nonylphenol using transcriptomic and phenotypic responses.

Identification of metabolic pathways in *Daphnia magna* explaining hormetic effects of Selective Serotonin Reuptake Inhibitors and 4-nonylphenol using transcriptomic and phenotypic responses.

Bruno Campos¹, Natàlia Garcia-Reyero², Claudia Rivetti¹, Lynn Escalon³, Tanwir Habib⁴, Romà Tauler¹, Stefan Tsakovski⁵, Benjamín Piña¹, Carlos Barata¹

¹Institute of Environmental Assessment and Water Research (IDAE-CSIC), Jordi, Girona 18, 08034, Barcelona, Spain.

²Institute for Genomics, Biocomputing and Biotechnology, Mississippi State University, Starkville, MS 39759, USA

³U.S. Army Engineer Research and Development Center, Environmental Laboratory, 3909 Halls Ferry Road, Vicksburg, MS 39180, USA

⁴Badger Technical Services, 12500 San Pedro Avenue, Suite 450, San Antonio, TX 78216, USA

⁵Department of Analytical Chemistry, Faculty of Chemistry, Sofia University, James Bourchier Blvd, 1164 Sofia, Bulgaria

Published: Environmental Science and Technology, 47, (2013), 9434-9443

Abstract

The molecular mechanisms explaining hormetic effects of Selective Serotonin Reuptake Inhibitors (SSRIs) and 4-nonylphenol in *Daphnia magna* reproduction were studied in juveniles and adults. Transcriptome analyses showed changes in mRNA levels for 1,796 genes in juveniles and 1,214 genes in adults (out of 15,000 total probes) exposed to two SSRIs (fluoxetine and fluvoxamine) or to 4-nonylphenol. Functional annotation of affected genes was improved by assuming the annotations of putatively homologous *Drosophila* genes. Self-Organizing Map analysis and Partial Least Square Regression coupled with selectivity ratio procedures analyses allowed to define groups of genes with specific responses to the different treatments. Differentially expressed genes were analysed for functional enrichment using Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes databases (KEGG). Serotonin metabolism, neuronal developmental processes, and carbohydrates and lipid metabolism functional categories appeared as selectively affected by SSRI treatment, whereas 4-nonylphenol de-regulated genes from the carbohydrate metabolism and the ecdysone regulatory pathway. These changes in functional and metabolic pathways are consistent with previously reported SSRI and 4-nonylphenol hormetic effects in *D. magna*, including a decrease in reserve carbohydrates and an increase in respiratory metabolism.

KEYWORDS: *Daphnia magna*, microarrays, mechanism of action, 4-nonylphenol, SSRIs, fluoxetine, fluvoxamine

4.1. Introduction

There is increasing evidence that the presence of many emerging pollutants in aquatic ecosystems may have detrimental effects on aquatic biota (Daughton and Ternes, 1999). In the last decade substantial research has been conducted evaluating toxic effects of pharmaceuticals and other emerging pollutants across non-vertebrate species (Fent *et al.*, 2006; Pal *et al.*, 2010). Among emerging pollutants, those that may act as putative endocrine disruptors in invertebrate species, for example altering moulting and reproductive processes, are of special concern (Barata *et al.*, 2004). The industrial detergent degradation product 4-nonylphenol and the pharmaceutical group of Selective Serotonin Reuptake Inhibitors (SSRIs) had hormetic consistent effects (i.e. increased) on offspring production and/or juvenile developmental rates in *D. magna* at low concentrations (i.e. 3-15µg/l), inhibiting those responses at higher concentrations (i.e. 100-125µg/l) (Baldwin *et al.*, 1997; Campos *et al.*, 2012b; Hansen *et al.*, 2008). Surveys in US have reported levels of 12-540ng/L of fluoxetine in surface waters and effluents (Kolpin *et al.*, 2002) but total concentrations of SSRIs in aquatic systems were measured in the range of 840ng/L (Vasskog *et al.*, 2008) to 3.2µg/L (Metcalfe *et al.*, 2010). 4-Nonylphenol levels in surface waters as high as 8µg/L has been reported in surface waters (Navarro *et al.*, 2009). Therefore the above mentioned hormetic effects occurred at environmental relevant concentrations.

SSRIs switch life-history responses towards higher food levels at limiting food conditions: juveniles develop faster reach maturity earlier and females produce more offspring (Campos *et al.*, 2012a). Such hormetic effects, however, have a fitness cost: females exposed to SSRIs have enhanced glycogen and aerobic metabolism, which translates into a lower tolerance to starvation at low oxygen levels, a common situation in the field (Campos *et al.*, 2012a). Exposure of *D. magna* adult females to low concentrations of 4-nonylphenol increases reproduction and decreases offspring size (Campos *et al.*, 2012b; LeBlanc *et al.*, 2000). These effects are maladaptive in *D. magna*, since smaller offspring take longer to grow, reach smaller adult size and produce less eggs at maturity (Hammers-Wirtz and Ratte, 2000).

The mechanisms of action of SSRIs studying phenotypic responses of *D. magna* exposed to SSRIs were analysed by studying effects on levels of lipids, carbohydrate, proteins, oxygen consumption rates, survival, and offspring production after exposure to SSRIs alone or with a serotonin antagonist (Campos *et al.*, 2012a). The results from this analysis show that SSRIs act in a similar way in *D. magna* than in humans by increasing serotonergic activity, but in doing so they alter physiological processes such as increase glycogen and aerobic metabolism. At high doses 4-nonylphenol causes embryo arrest in *D. magna*, an effect that according to LeBlanc *et al.*, (2000) may be related to altered metabolism of endogenous steroids (such as ecdysone), resulting in perturbations in their provision to the newly produced eggs.

The above mentioned studies support the view that 4-nonylphenol and SSRIs increase offspring production through different mechanisms of action (Campos *et al.*, 2012a; Campos *et al.*, 2012b). SSRIs affect life-history responses of both, juveniles and adults, having important metabolic effects that in adults result in increased carbohydrate and aerobic metabolism. Sublethal effects of 4-nonylphenol have only been observed in adults and probably are related to altered metabolism of endogenous steroids like ecdysone (LeBlanc *et al.*, 2000).

The water flea *Daphnia magna* is possibly the invertebrate system most used in toxicology and experimental ecology and together with its close relative *D. pulex* is used as a model for environmental genomics research (Piña and Barata, 2011). *D. pulex* genome has been fully sequenced and about 50% of its genome is annotated (Colbourne *et al.*, 2011), thus that of its close relative *D. magna*, despite of being incomplete, may benefit from the former. Nevertheless, gene annotation available for the *D. pulex* genome was insufficient to aid functional analysis, thus additional annotation for functional enrichment and identification of gene networks and metabolic pathways can be performed using available bioinformatic tools such as Blast, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Asselman *et al.*, 2012; Garcia-Reyero *et al.*, 2009; Poynton *et al.*, 2012; Vandenbrouck *et al.*, 2010).

The main objective of this work is the study of molecular mechanisms explaining the hormetic effects observed in our two previous studies (Campos *et al.*, 2012a; Campos *et al.*, 2012b). This was accomplished using

transcriptional stress responses of *D. magna* juveniles and adults exposed to sublethal doses of 4-nonylphenol and the SSRIs fluoxetine and fluvoxamine. We aimed to identify gene pathways that characterize observed physiological and phenotypic response to these contaminants. To this effect, we used a custom microarray that has been successfully used to assess mechanisms of action of different pollutants in *D. magna* (Garcia-Reyero et al., 2012). Differentially transcribed genes were related to effects observed on offspring production of females chronically exposed to these pollutants using Partial Least Square (PLS) regression coupled with selectivity ratio (SR) procedures. Existing information from *D. pulex* and *Drosophila melanogaster* genomes was used to improve the currently poor annotation of *D. magna* genes and to facilitate the functional analyses of the observed transcriptomic changes.

4.2. Experimental Section

4.2.1. Chemicals

4-nonylphenol (CAS No 104-40-5, PESTANAL® analytical grade standard, 98.4% purity, Riedel-de-Haen, Germany); fluoxetine hydrochloride (CAS-No 56296-78-7; analytical standard, purity 100%) Sigma-Aldrich, USA), and fluvoxamine maleate (CAS-No 61718-82-9, analytical standard, purity 100%) were purchased from Sigma-Aldrich (USA/Netherlands). All other chemicals were analytical grade and were obtained from Merck (Germany).

4.2.2. Analytical Chemistry

Methods and measured concentrations of the studied chemicals in adult reproduction tests were reported elsewhere (Campos et al., 2012b) and in all cases were within 90% of nominal values indicating that degradation/adsorption/uptake to experimental vessels/animals were negligible.

4.2.3. Experimental animals

All experiments were performed using a well-characterized single clone of *D. magna* (Clone F), maintained indefinitely as pure parthenogenetic cultures (Barata and Baird, 2000). Individual or bulk cultures of 10 animals/L were maintained in ASTM hard synthetic water as described in (Barata and Baird,

1998). Individual or bulk cultures were fed daily with *Chorella vulgaris* Beijerinck (5×10^5 cells/mL, respectively, corresponding to $1.8\mu\text{g C/mL}$). *C. vulgaris* was grown axenically in Jaworski/Euglena gracilis 1: 1 medium (CCAP, 1989). The culture medium was changed every day, and neonates were removed within 24h. Photoperiod was set to 14h light: 10h dark cycle and temperature at $20 \pm 1^\circ\text{C}$.

4.2.4. Experimental design

Juveniles and adult females from two distinct assay-experiments were considered for transcriptomic studies. Assays with adult females were conducted in a previous study (Campos *et al.*, 2012b) and are briefly described in experiment 2. Selected exposure levels of the studied compounds include those treatments that in a previous study (Campos *et al.*, 2012b) affected most reproduction responses: for SSRIs, $7\mu\text{g/L}$ of FV and $40\mu\text{g/L}$ of FX; for 4-nonylphenol 20 and $60\mu\text{g/L}$.

Experiment 1: 72 h exposures of juveniles to the SSRIs: FV ($7\mu\text{g/L}$), FX ($40\mu\text{g/L}$) and the industrial detergent 4-nonylphenol (20, $60\mu\text{g/L}$). Groups of twenty juveniles (less than 24h old) were exposed to selected exposure levels in 0.5L of ASTM hard water with food present (5×10^5 cells/mL of *C. vulgaris*) during two consecutive moults (about 3 days at 20°C and high food levels) (Nogueira *et al.*, 2004b). Longer exposure periods were not performed as juveniles enter the adolescent instar where ovaries start to develop (Ebert, 1994). Contaminants were dosed using acetone as a carrier (0.1ml/L). A carrier control of 0.1ml/L was also used to serve as a baseline comparison of transcriptomic responses. Treatments were quadruplicated. At the end of exposure the 20 juveniles of each treatment *per replicate* were collected, pooled in an eppendorf vial, flash- frozen in liquid N₂ and preserved at -80°C until RNA extraction.

Experiment 2: Adult reproduction tests were performed to determine the effects of the chemicals of interest on transcriptomic responses and reproduction rates on adult stages (Campos *et al.*, 2012a). Ten gravid females were separately exposed to the same concentrations of 4-nonylphenol (20, $60\mu\text{g/L}$), fluvoxamine ($7\mu\text{g/L}$) and fluoxetine ($40\mu\text{g/L}$) as described in experiment 1 using a food ration of 5×10^5 cells/mL of *C. vulgaris*. Likewise in

experiment 1 all contaminants were dosed in the carrier acetone (0.1mL/L) and a solvent control treatment of acetone (0.1mL/L) was also included for baseline comparison of transcriptomic responses. Experiments started with 8-9 day old gravid females, which were exposed during three consecutive broods to the studied chemicals (10-14 days). Cultures of 100 to 150 individuals (<24h old neonates) were initiated and maintained in bulk cultures as described above. Within 24h of deposition of the first clutch into the brood chamber, single females were removed and randomly assigned to each treatment. The first batch of neonates (hatching within the first 48-72h) was always discarded and not evaluated, as these animals were not exposed to the tested chemicals during their entire developmental period. Thus, only neonates from the second, third and fourth broods were counted for assessing effects on total offspring production. Just after releasing their fourth clutch into the brood pouch, eggs were gently flushed from the brood pouch and females were immediately flash-frozen in liquid N₂ and preserved at -80°C until RNA extraction. This protocol, by using only de-brooded females excludes the contribution of developing embryos on transcriptomic responses. Furthermore, the use of de-brooded adults in the first hours of their intermolt instar ensured measurement of transcriptomic responses of all the studied females at the beginning of the intermolt cycle, thus minimizing undesirable variation of gene transcription patterns within females across the molt cycle (Piña and Barata, 2011).

4.2.5. RNA extraction

Total RNA was isolated from the samples using Trizol reagent® (Invitrogen, Carlsbad, USA) following the manufacturer's protocol and quantified in a NanoDrop D-1000 Spectrophotometer (NanoDrop Technologies, Delaware, DE). RNA quality was checked in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). The samples that did not show degradation were used for microarray analysis.

4.2.6. Microarrays

Custom *D. magna* 15,000 probe microarrays (GLP13761) were purchased from Agilent (Palo Alto, CA, USA). A total of four replicates per treatment were used for both juveniles and adult exposures. One µg of total RNA was used for

all hybridizations, and cDNA synthesis, cRNA labelling, amplification, and hybridizations were performed following the manufacturer's kits and protocols (Quick Amp labelling kit; Agilent, Palo Alto, CA). The Agilent one-color Microarray Based Gene Expression Analysis v6.5 was used for microarray hybridizations according to the manufacturer's recommendations. An Agilent high-resolution C microarray scanner was used to scan microarray images. Data were resolved from microarray images using Agilent Feature Extraction software v10.7. Raw microarray data from this study have been deposited at the Gene Expression Omnibus Web site (www.ncbi.nlm.nih.gov/geo/) with accession number GSE45053.

Table 4.1. Primer pairs designed from existing sequences used for amplification of selected *Daphnia magna* partial gene sequences.

Genes	Primer sequence		
	forward	Reverse	Amplic
G3PDH	GACCATTACGCTGCTGAATACG	CCTTGCTGACGCCGATAGG	100
Acon	AACTAACAAACGGCACTGGCAC	CTTCTCCGTTAGCGCCTTG	81
Ddc	AATGATTCCCTGAAGCCGCC	CCAGGCATAATGACCGCGTT	81
Idh	CTGTTTCCGCGAACCTATCC	GACGACCAATGACAATGGGC	81
ATPCL	AACTTGCGGCACATGAGCT	CTTGTGCCGATGCCGAAC	81
Thiolase	AGGCACACGCAATGTTCC	TTGGCCGTGCTAACGATG	81
faa	ACTGGAACCCCACCTGGAGT	AACTTCGATTCACCAACCGT	81

4.2.7. Validation of Microarray results by qRT-PCR

Microarray results were validated with real-time quantitative polymerase chain reaction (qPCR). We selected six significantly regulated genes from different pathways/gene families: lipids metabolism (thiolase), Krebs cycle (aconitase-acon, isocytrate dehydrogenase-idh, ATP citrate lyase-ATPCL), tryptophan metabolism (dopamine decarboxylase-Ddc) and nucleotide metabolism (fumarilacetoacetate-Faa). The gene G3PDH (glyceraldehyde 3-phosphate dehydrogenase) was used as internal control. For each of these genes primers were designed with Primer Quest (IDT Technologies, Coralville, IA) and are listed in Table 4.1. Quantities of 1µg were retrotranscribed to cDNA using First Strand cDNA Synthesis Kit Roche® (Germany) and stored at -20°C. Aliquots of 10ng were used to quantify specific transcripts in Lightcycler® 480 Real Time PCR System (Roche, Germany) using Lightcycler 480 SYBR Green I Master® (Roche, Germany). Relative abundance values of all genes were calculated from the second derivative of their respective amplification curve, Cp

values calculated by technical triplicates. Cp values of target genes were compared to the corresponding reference gene (Pfaffl, 2001).

4.2.8. Gene Expression analysis

Microarray data were analysed using GeneSpring GX v4.0 software (Agilent). Data were normalized using quantile normalization and baseline transformation to the median of all samples. Differentially transcribed genes of *D. magna* individuals exposed to 4-nonylphenol and SSRIs from that of carrier controls (hereafter named as controls) were analysed using one way ANOVA followed by Dunnett's pos-hoc comparison tests and filtered based on expression levels ($p<0.05$; cut-off 1.5 fold change). A list of differentially transcribed genes with annotation for all the treatments can be found in Table 4.2 available at <http://pubs.acs.org/doi/suppl/10.1021/es4012299> and included in supplementary electronic material).

Transcriptomic patterns of differentially expressed genes of juveniles and adults were analysed using the Multi-Experiment viewer MeV4 software (Saeed *et al.*, 2003), both by hierarchical clustering (sites) and Self Organizing Map (SOM) analysis (features), and the standard normalization protocol. Furthermore, the assays performed with single adult females (experiment 2), allowed to establish possible correlations between individual transcriptomic profiles and the observed effects of both SSRIs and 4-nonylphenol treatments on *D. magna* reproduction responses, and hence the identification of putative gene regulatory pathways affecting these reproduction responses. Partial Least Squares Regression (PLS) was used to investigate the correlations between transcriptome data (X-variables) and total offspring production (y variable) under both SSRIs and 4-nonylphenol treatments. Since both treatments affected fecundity similarly (increased offspring production) but likely through different molecular mechanisms, SSRIs and 4-nonylphenol treatments were analysed separately by PLS-SR. Genes contributing more to the prediction of the reproduction responses by the proposed PLS model were selected using the selectivity ratio (SR) (Rajalahti *et al.*, 2009). This variable selection method minimizes the effect of uncorrelated X variance sources. Those genes (X-variables) with SR values higher than the mean were considered to be the most relevant for explaining the Y responses related to treatments. Prior to data

analysis, X and Y variables were mean-centred to correct for their offset. The number of PLS-SR components was finally set at two, according to cross validation by the leaving-one-out prediction errors' criteria (Wold *et al.*, 2001). PLS-SR analyses were performed using the PLS Toolbox version 6.5, Eigenvector Research Inc. (Manson, WA) running under Matlab 7.4 computer environment, (MathWorks, Natick, Massachusetts)

Genes included in the clusters identified by SOM and PLS-SR analyses were annotated using the recent update of the custom microarray, which incorporates *D. pulex* sequence annotations available at the Fleabase (Garcia-Reyero *et al.*, 2012). Further annotation was performed by identifying putative homologous *Drosophila melanogaster* genes using BLAST at Flybase server (<http://flybase.org/blast/>). Annotated genes were ascribed to functional Gene Ontology (GO terms) and to metabolic pathways using the AmiGO! Term Enrichment tool (<http://www.geneontology.org/>) and the Kyoto Encyclopaedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/kegg2.html>) databases, respectively.

4.3. Results

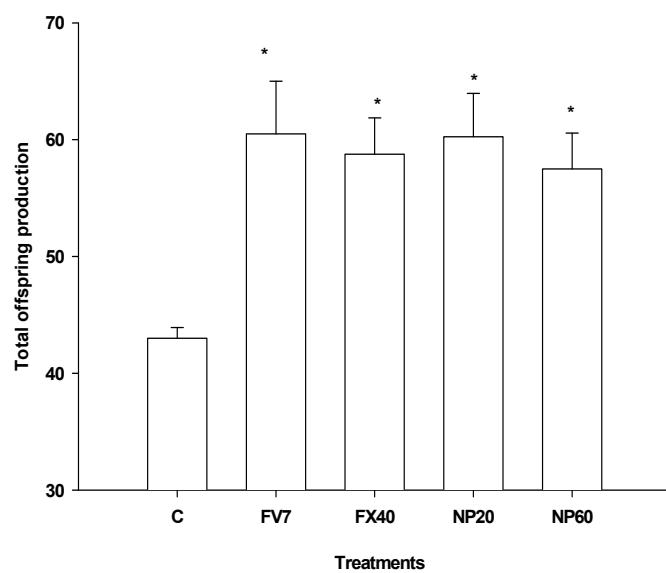


Figure 4.1. Total offspring production (Mean \pm SE, N=4) of the four selected females exposed to the studied SSRIs and 4-nonylphenol treatments. C, FV10, FX40, N20, N60 are solvent control, fluvoxamine at 7 μ g/L, fluoxetine at 40 μ g/L, 4-nonylphenol at 20 μ g/L and 60 μ g/L, respectively. Asterisks indicate significant differences from solvent controls following ANOVA and Dunnett's comparison tests

4.3.1. Phenotypic responses

There was no mortality during juvenile exposures. Selected females for transcriptomic analyses produced significantly ($P<0.01$; $F_{4,15}=5.02$) more offspring than controls in all treatments (Figure 4.1).

4.3.2. Transcriptome analysis

Transcriptome responses of juveniles and adults of experiments 1 and 2 were analysed separately. A total of 1,796 and 1,214 genes were identified as differentially transcribed in at least one of the chemical treatments, in juveniles and adults, respectively. From the above-mentioned genes, only 221 were annotated (Table 4.2 available at <http://pubs.acs.org/doi/suppl/10.1021/es4012299> and included in supplementary electronic material).

Hierarchical clustering separated most biological replicates of SRRIs, 4-nonylphenol and control treatments on both the juvenile and adult transcriptomic data set (Figure 4.2A and B). The SOM analysis performed on the differentially transcribed genes in juveniles and adults revealed six clusters of significantly de-regulated genes (Fig 4.3). For juveniles cluster 1 include 151 significantly ($P<0.01$) up-regulated genes by both SRRI compounds (FX and FV). Clusters 2 and 3 included 210 and 456 FV down-regulated ($P<0.01$) unique genes, respectively. For adults, cluster 4 included 32 genes significantly ($P<0.01$) down-regulated by both SSRIs (FV and FX treatments), cluster 5 included 86 genes significantly ($P<0.01$) down-regulated by 4-nonylphenol treatments (20, 60 μ g/L), whereas cluster 6 included 19 genes up-regulated by 4-nonylphenol treatments (Fig 4.3). SOM graphs together with the de-regulated genes for each cluster are depicted in Figure 4.3 and Table 4.3. (Table 4.3 is available at <http://pubs.acs.org/doi/suppl/10.1021/es4012299> and provided as supplementary electronic material).

PLS analyses were performed separately for the different treatments considered (control and SSRIs) and (control and 4-nonylphenol treatments). In each data set the 1,214 de-regulated gene fragments were selected as predictors (X matrix of predictor variables) and the total offspring production of the exposed females was used as predicted variable (Y vector of predicted variables). The two components introduced in the PLS analysis explained

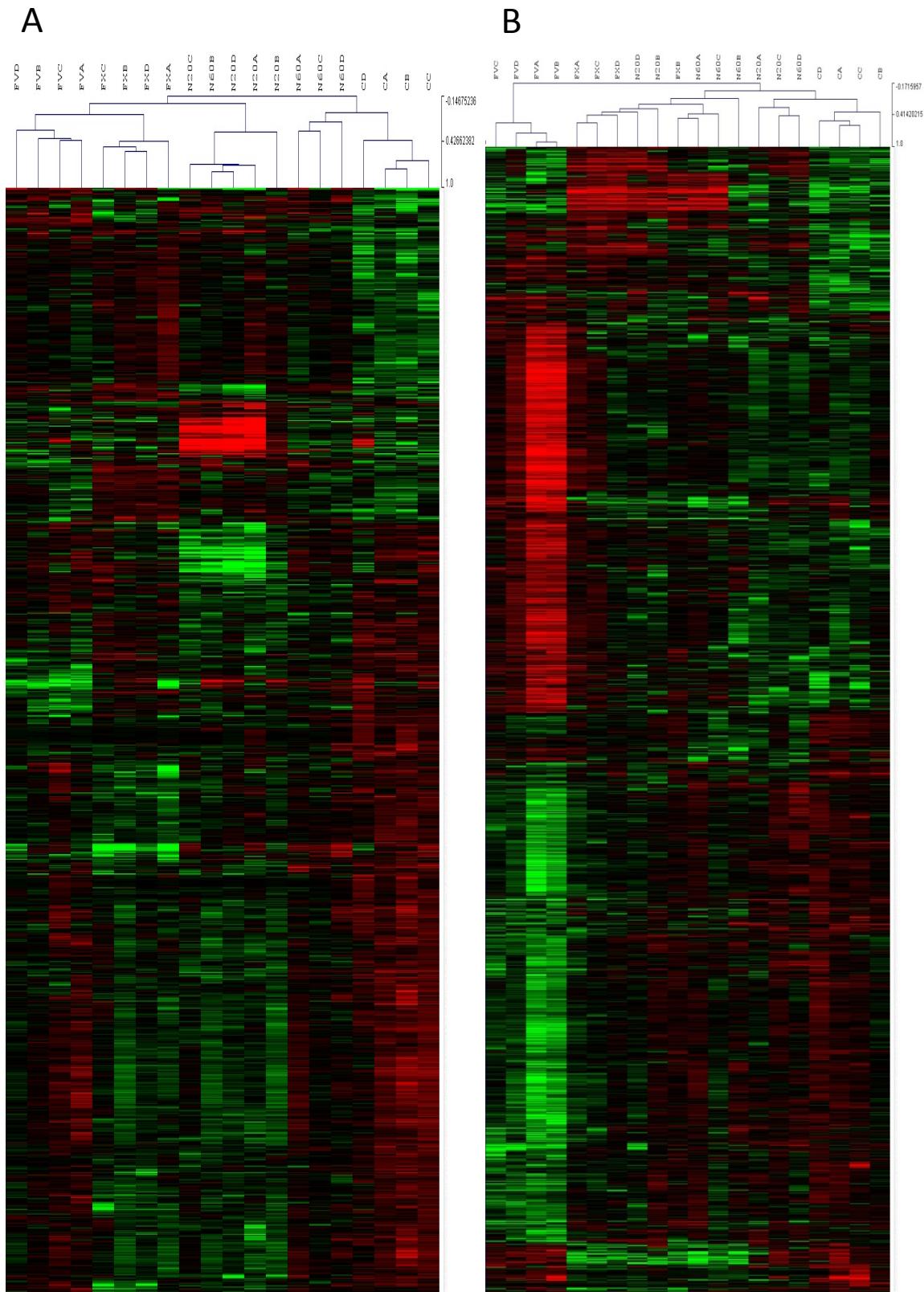


Figure 4.2. Heat map and hierarchical clustering (Pearson correlation) base on log2 ratios of the differentially transcribed genes in *Daphnia magna* adults (A) and juveniles (B) across the experimental replicates for the SSRIs (FV, FX) and nonylphenol (N20, N60) treatments. The replicates are identify as A,B,C,D. Gene transcripts in red and green are up and down regulated and those in black unchanged. Colour scale spans from -2.5 to 2.5.

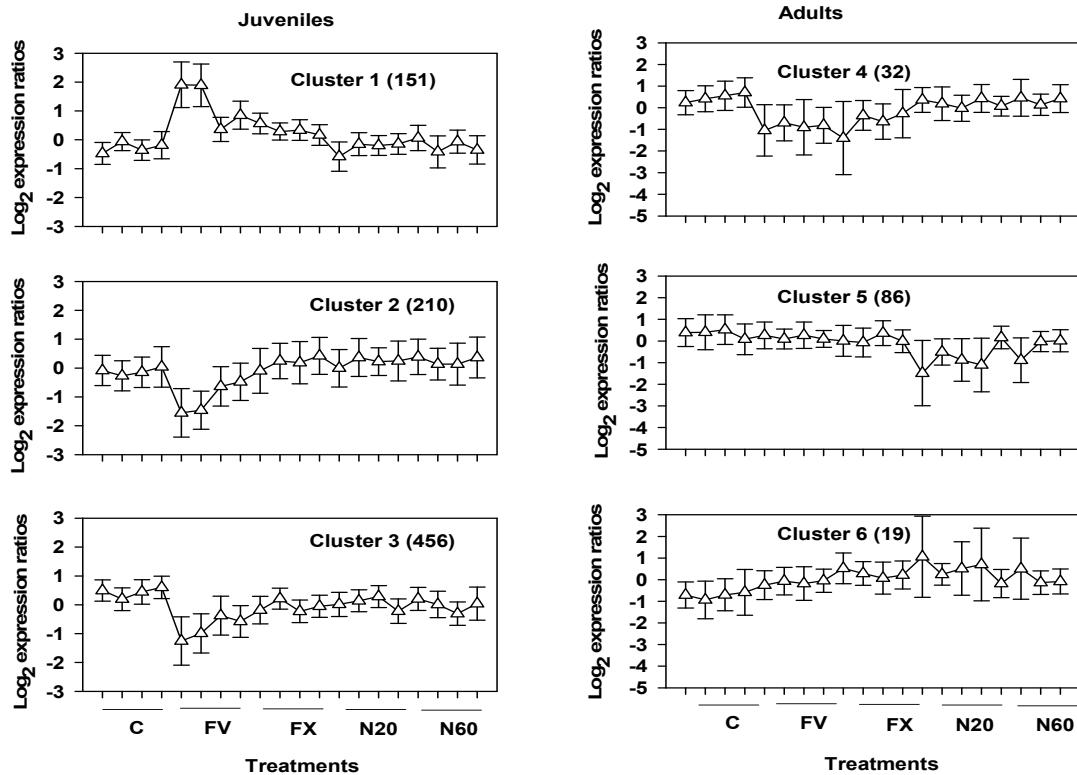


Figure 4.3. Self-Organizing Maps (SOM) of gene transcription responses of juveniles and adults across the experimental replicates for SSRIs (fluvoxamine, FV; fluoxetine, FX) and 4-nonylphenol (N20, N60) treatments. Results are depicted as Mean \pm SD of log₂-ratios. Number of de-regulated genes are depicted between parenthesis.

around 80% of the total Y variance ($r^2 = 0.79$) for the prediction of total offspring production of females treated either with SSRIs (Figure 4.4A) or with 4-nonylphenol (Figure 4.4B). Following the established SR cut-off criteria, PLS analysis identified 272 and 331 genes differentially affected by SSRIs and 4-nonylphenol, respectively (Figure 4.4). These predictor genes formed two differentiated groups: one in close proximity with SSRIs or 4-nonylphenol Y-treatment including genes that co-varied positively with both treatments, and a second one close to control treatments and including genes inversely correlated with the two studied treatments. The final set of annotated genes differentially affected by SSRIs and 4-nonylphenol treatments (35 and 31 genes, respectively) are identified by numbers in Figure 4.4. Correlation plots between the log₂ of fold change ratios of the selected annotated and non-annotated genes with the total offspring production are given in Table 4.4.

Table 4.4. Annotated and non-annotated genes selected in the PLS-SR analyses performed for SSRIs and 4-nonylphenol data. For annotated genes their name and associated cod number assigned in Fig 4.4 are reported. Only the probe name of non-annotated genes having significant ($p<0.05$) correlation coefficients of their relative expression with total offspring are depicted. * $0.05 < P < 0.01$; ** $P < 0.01$, N=12. Full table in annex file.

SSRI's			4 nonylphenol				
Name	Cod	Correlation	Name	Cod	Correlation		
mt:ND1	35	-0.79	**	Got1	25	-0.82	**
Tom7	53	-0.76	**	cdc16	8	-0.76	**
Rfabg	40	-0.75	**	Acon	1	-0.76	**
CG7718	15	-0.7	*	CG16986	10	-0.76	**
Spn2	46	-0.7	*	Cht6	17	-0.76	**
cher	16	-0.7	*	UGP	56	-0.72	**
san	43	-0.69	*	CG33123	12	-0.71	**
Aph-4	4	-0.67	*	CG3368	13	-0.7	*
EndoA	19	-0.67	*	Sr-CI	48	-0.69	*
Papss	38	-0.64	*	Oda	37	-0.69	*
tok	52	-0.62	*	AnnIX	3	-0.68	*
GlyP	24	-0.59	*	mRpS9	32	-0.67	*
spen	45	-0.54		mt:ND1	34	-0.67	*
jdp	28	-0.54		Btk29A	6	-0.67	*
MstProx	33	-0.54		Papss	38	-0.63	*
spok	47	-0.52		CG42337	14	-0.63	*
Sr-CI	48	-0.51		Rfabg	40	-0.59	*
Act57B	2	-0.51		san	43	-0.59	*
Eps-15	20	-0.5		fbp	21	0.58	*
Arf12F	5	-0.47		KdelR	29	0.58	*
sns	44	-0.45		CG18155	11	0.66	*
dy	18	0.49		Rab11	39	0.69	*
ninaG	36	0.49		Sac1	42	0.7	*
Try29F	55	0.55		RhoGAP1A	41	0.72	**
CadN	7	0.6	*	flr	22	0.74	**
Sac1	42	0.62	*	Hil	26	0.75	**
Tpc2	54	0.64	*	βTub56D	59	0.76	**
yin	58	0.66	*	Vha55	57	0.77	**
Gasp	23	0.67	*	cenG1A	9	0.81	**
Mcm5	31	0.67	*	Try29F	55	0.81	**
βTub56D	59	0.69	*	Tg	50	0.84	**
128up-PA	60	0.71	*				
Rab11	39	0.76	**				
Kr-h1	30	0.77	**				
Idh	27	0.84	**				

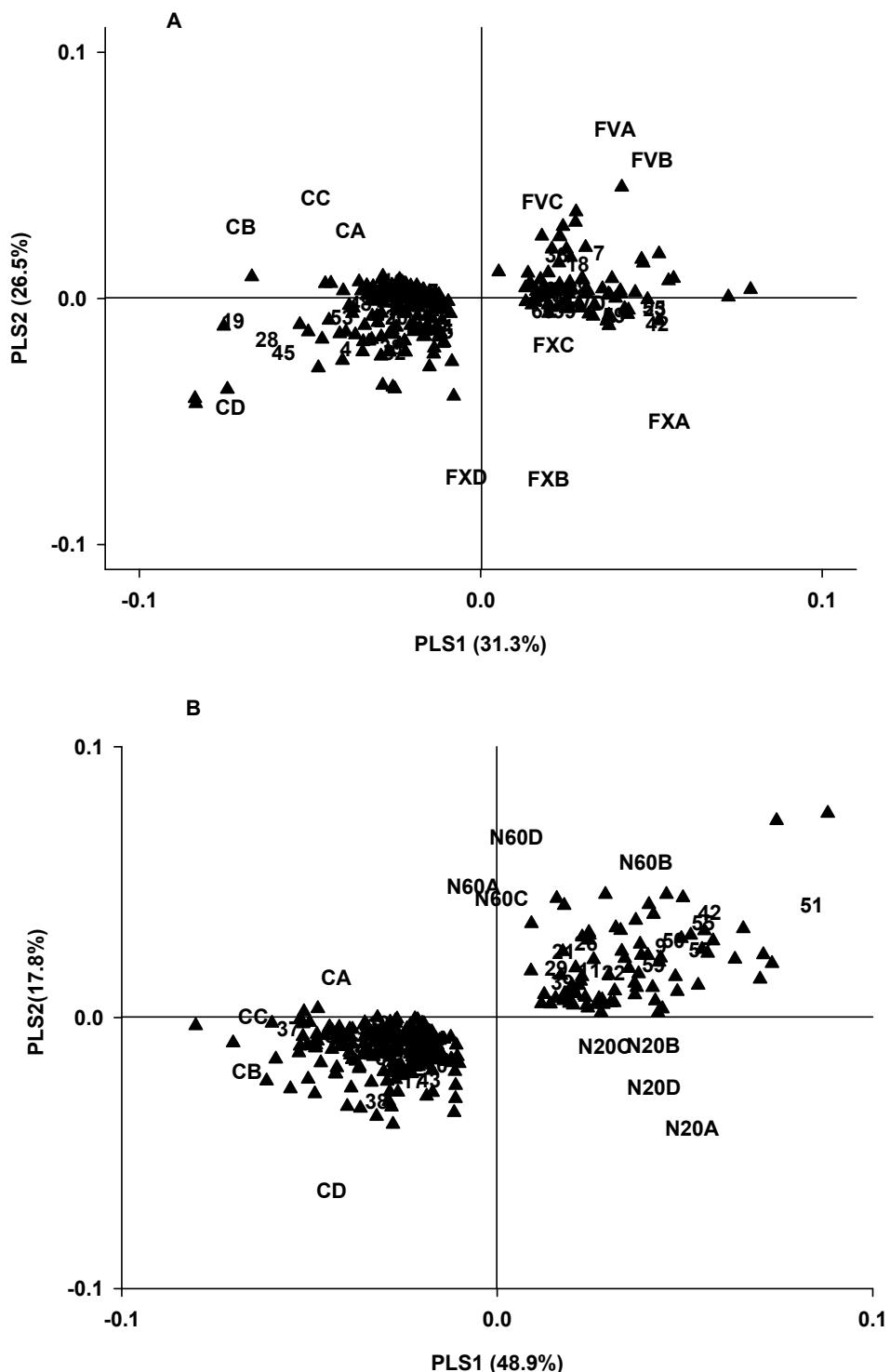


Figure 4.4. PLS-SR summary of results for the two different sample treatments (Figure 1A, SSRIs; Figure 1B, 4-nonylphenol) using differential expressed genes as predictors (X) and total offspring production as response (Y). First and second PLS bi-plots showing selected gene loadings (triangles) and offspring production scores (sample identification letters). Only genes with a selectivity ratio above the mean were considered. Annotated genes are indicated by numbers described in the Table 4.4. The four replicates are identified as A,B,C,D.

Results from RT-qPCR confirmed the fold change ratios of mRNA levels observed with the microarray, both in magnitude and direction, for the six selected genes (Figure 4.5) with a Pearson correlation coefficient of 0.83 ($P<0.01$, $N=33$).

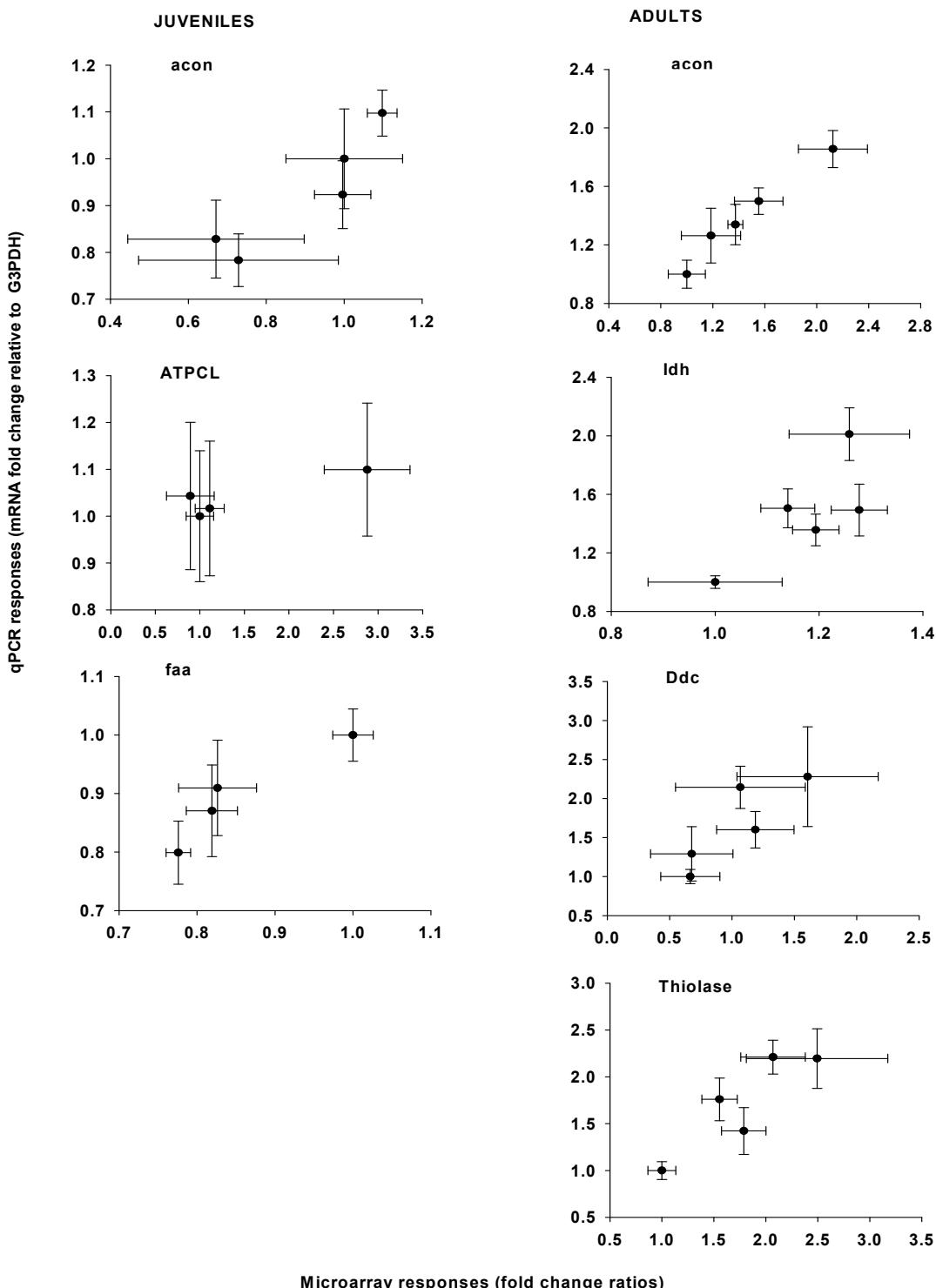


Figure 4.5. Confirmation of the array results with qPCR. Array and RT-qPCR results are represented as gene fold change ratios. Errors bars are standard errors of mean (SE, $N=4$).

GO analyses were performed on annotated genes from SOM and PLS-SR clusters (Table 4.5, further details of GO terms and the name of involved genes are depicted in Table 4.6 that is available at <http://pubs.acs.org/doi/suppl/10.1021/es4012299> and provided as supplementary electronic material). Juveniles appeared as selectively more affected by SSRIs treatments than adults, showing the highest amount of annotated genes grouped into GO term ($P<0.05$; 151 genes in total, Table 4.5).

Adult SOM and PLS-SR clusters affected by SSRIs included 40 genes that could be adscribed to specific GO term (Table 4.5). 4-Nonylphenol only affected adult SOM and PLS-SR clusters, which included 38 genes with a specific GO term assignation (Table 4.5). The GO terms with the highest number of de-regulated genes were related to three parental GO categories: developmental processes, structural constituents and binding (Table 4.5). Developmental processes were only affected by SSRIs. Other processes only affected by SSRIs were synaptic vesicle transport, localization and response to stimuli. Structural constituents, binding and catalytic activity related GO categories were shared by both SSRIs and 4-nonylphenol treatments although most of the de-regulated genes and sub-GO categories belonged to SSRIs treatments. The only unique GO category affected by 4-nonylphenol was lipid binding.

Metabolic pathway analyses of de-regulated genes (>1.5 fold) in adult females exposed chronically to SSRIs and 4-nonylphenol indicated that 20 of these genes regulated metabolic pathways (Figure 4.6). More specifically, SSRIs and 4-nonylphenol de-regulated 12 and 7 genes from metabolism of carbohydrates and lipids and Krebs cycle, respectively. Further details of KEGG analyses are in Table 4.7.

Table 4.5. GO terms for the obtained six SOM and two PLS gene clusters for SSRI's and 4-nonylphenol (N) treatments. SOM clusters of juveniles (JUV 1, 2, 3) and of adults (ADT 4,5,6) and PLS ones for SSRIs and 4-nonylphenol are depicted in Fig 4.3, Fig 4.4. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Further information of GO terms and annotated genes belonging to each group are in Table 4.6). Arrows indicate the direction of de-regulation.

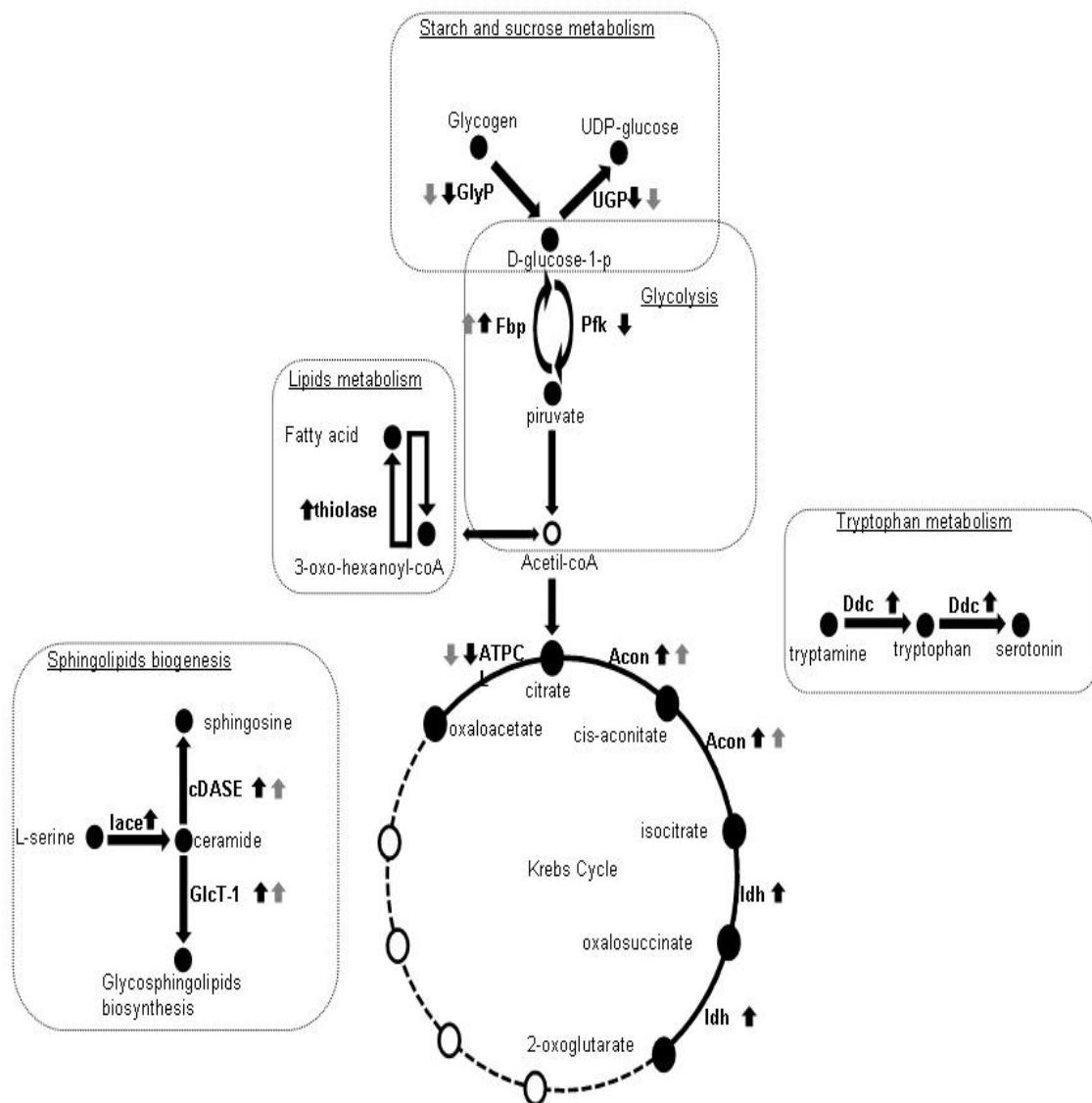


Figure 4.6. KEGG diagram of metabolic pathways with the genes up and down regulated across SSRIs (in bold) and 4-nonylphenol (in grey) treatments in adult females. Arrows indicated de-regulation direction relative to control treatments. Further details of genes are in Table 4.7.

Table 4.6. Genes with their mean fold change ratios that were included in the metabolic KEGG paths of Fig 4.6. FV7, FX40, N20 and N60 are fluvoxamine, fluoxetine and 4-nonylphenol at 7, 40, 20 and 60µg/L, respectively.

Gene	Name	Probe	Fold change ratios							
			FV7	FX40	N20	N60				
Acon	Aconitase (EC:4.2.1.3)	DM01751P1	1.19	up	2.13	up	1.55	up	1.37	up
ATPCL	ATP citrate lyase (EC:2.3.3.8 6.2.1.5)	DM02886P2	1.4	down	2.71	down	2.09	down	1.56	down
CDase	Ceramidase (EC:3.5.1.23)	DM01115P2	1.34	up	2.33	up	1.65	up	1.78	up
GlcT-1	CG6437 gene product from transcript CG6437-RA (EC:2.4.1.80)	DM00695P3	1.5	up	2.15	up	2.05	up	1.48	up
Got1	Glutamate oxaloacetate transaminase 1 (EC:2.6.1.1)	DM02714P3	1.76	down	2.16	down	2.47	down	1.95	down
Papss	PAPS synthetase (EC:2.7.1.25 2.7.7.4)	DM00609P1	1.21	up	1.7	up	1.32	up	1.22	up
UGP	CG4347 gene product from transcript CG4347-RE (EC:2.7.7.9)	DM02451P3	1.49	down	1.9	down	2.09	down	1.87	down
Vha55	Vacuolar H ⁺ -ATPase 55kD B subunit (EC:3.6.3.14 3.6.3.6)	DM01785P2	1.54	up	3.11	up	3.19	up	3.53	up
awd	abnormal wing discs (EC:2.7.4.6)	DM02782P4	1.42	down	2.26	down	2	down	1.61	down
fbp	fructose-1,6-bisphosphatase (EC:3.1.3.11)	DM01662P1	1.34	up	1.83	up	1.63	up	1.95	up
GlyP	Glycogen phosphorylase (EC:2.4.1.1)	DM00386P3	3.22	down	1.8	down	1.93	down	1.74	down
r	rudimentary (EC:2.1.3.2 3.5.2.3 6.3.4.16 6.3.5.5)	DM01003P2	1.13	down	1.9	down	2.22	down	1.68	down
Aph-4	Alkaline phosphatase 4 (EC:3.1.3.1)	DM02798P2	4.21	down	1.71	down	2.16	down	1.35	down
Ddc	Dopa decarboxylase (EC:4.1.1.28)	DM02360P4	1.07	up	1.61	up	1.19	up	1.48	down
Gdh	Glutamate dehydrogenase (EC:1.4.1.2 1.4.1.3)	DM04415P1	2.03	down	1.1	up	1.05	down	1.24	down
Idh	Isocitrate dehydrogenase (EC:1.1.1.42)	DM01625P3	2.28	up	2.26	up	1.14	up	1.19	up
Pfk	Phosphofructokinase (EC:2.7.1.11)	DM02942P2	1.18	up	2.31	down	1.36	down	1.07	up
Thiolase	CG4581 gene product from transcript CG4581-RA (EC:1.1.1.211 2.3.1.16)	DM01778P1	1.55	up	2.49	up	1.07	up	1.29	up
lace	CG4162 gene product from transcript CG4162-RA (EC:2.3.1.50 2.6.--)	DM02843P3	2.18	up	2.05	up	1.47	up	1.24	up

4.4. Discussion

In this study we tested the hypothesis that low concentrations of SSRIs and 4-nonylphenol affected offspring production and/or juvenile developmental rates through different mechanisms of action. Our hypothesis was tested by combining two developmental stages, juveniles and adults, exposed during two (3 days) and three (10 days) consecutive instars, respectively. Although in absolute terms juvenile exposures were much shorter than that of adults, both schemes included physiological equivalent periods (i.e. include 2-3 instars) (Nogueira *et al.*, 2004a). Previous results also indicate that sublethal effects on juvenile developmental rates of SSRIs occurred at similar concentrations than those of adults (Campos *et al.*, 2012a). Therefore, we consider that juvenile and adult stages were chronically exposed to equivalent concentrations and periods in our experiments.

Hierarchical clustering clearly grouped replicated samples of SSRIs, 4-nonylphenol and control by treatment in both juvenile and adult exposures, providing the first evidence that these contaminant families induced specific and unique transcriptional patterns. The number of de-regulated genes and significant GO terms obtained for juveniles exposed to all three chemicals was higher than that of adults, which supports the hypothesis that short term exposures may be more suited to assess differentially altered transcriptomic responses. This was particularly evident for the SOM clusters obtained for adults that only involved 17 annotated genes whereas those of juveniles included 151 ones (Table 4.5). Alternatively, the number of de-regulated genes could be a direct consequence of previously reported phenotypic effects. Previous work indicated that at the studied exposure levels FV affected juvenile developmental rates whereas FX and 4-nonylphenol did not (Campos *et al.*, 2012b). This is consistent with the apparent stronger effect of FV on the juveniles' transcriptome, both in terms of number of de-regulated genes and of the number of significantly enriched GO terms (Fig 4.2A and B). Reproduction was similarly affected by the three chemicals at all exposure levels, and so was the number of annotated genes and enriched GO terms defined by both SOM and PLS-SR analyses (7,10 genes in SOM clusters for SSRIs and 4-

nonylphenol, respectively; 35 and 32 genes selected by PLS-SR for SSRIs and 4-nonylphenol, respectively; Table 4.5).

Relating molecular responses to phenotypic effects is crucial in environmental risk assessment. A promising approach is the adverse outcome pathway (AOP) framework, which is a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organization relevant to risk assessment (Ankley *et al.*, 2010). The application of AOP has been restricted to model organisms (i.e. few fish species) (Ankley *et al.*, 2009; Tanneberger *et al.*, 2013; Yozzo *et al.*, 2013). In a previous study (Campos *et al.*, 2012b) we showed that 4-nonylphenol increased offspring production but decreased at the same time offspring size. According to several studies being smaller at birth bears a fitness disadvantage since smaller neonates are more sensitive to environmental stress, and have lower fitness than bigger ones (Barata *et al.*, 2001; Hammers-Wirtz and Ratte, 2000). SSRIs also increased offspring production with apparently no effects on offspring size but such effects were related with increasing oxygen demand and being less tolerant to low oxygen levels (Campos *et al.*, 2012a). Thus in the two studied chemical groups (4-nonylphenol and SSRIs), adverse effects were directly or indirectly associated with increasing reproduction at the selected exposure levels. In the present study the combination of further annotation establishing gene homologies with known genomes like *D. melanogaster* improved the functional enrichment of de-regulated *D. magna* gene sequences and hence it was possible to obtain reliable gene pathways using GO and KEGG. Furthermore, the use of multivariate methods like PLS and SR to identify de-regulated genes related with observed phenotypic effects in chronic exposures performed with adult females identified 111 annotated genes related to effects observed in reproduction. More standard clustering methods like SOM only allowed to identify 17 de-regulated genes in adult females. PLS methods are already being used to relate gene transcription patterns with toxicological effects (Dom *et al.*, 2012). The combination of PLS with SR, however, offers the possibility to select de-regulated genes highly correlated with phenotypic responses (see Table 4.4). Juvenile SOM clusters included several differentially expressed genes involved in developmental processes after SSRIs exposure, which can be linked

to the reported increased juvenile developmental rates (Campos *et al.*, 2012b). The SSRIs exposures also de-regulated ribosomal structural constituents, which could be linked to protein biosynthesis or cell proliferation. (Dom *et al.*, 2012) reported the up-regulation of ribosomal constituents related genes in *D. magna* exposed to polar narcotics as intent to overcome toxicant stress and /or to restore the damage elicited by exposure to those narcotics. Neuronal pathways, including synaptic vesicle transport, establishment and maintenance of localization, and response to stimuli were GO functional terms down-regulated in both juvenile and adults. The down regulation of neuronal pathways may be associated to an adaptive gene response to the presence of neuronal active compounds, as a similar response has been observed for the neurotoxic insecticide methomyl (Pereira *et al.*, 2010).

Three genes involved in the ecdysone regulatory pathway that controls morphogenesis and integrin expression during *Drosophila* metamorphosis (D'Avino and Thummel, 2000) appeared specifically down-regulated by 4-nonylphenol treatment: papss (3'-phosphoadenosine 5'-phosphosulfate synthetase), got1 (Glutamate oxaloacetate transaminase), and btk29A (Bruton's tyrosine kinase). This is consistent with the hypothesis that 4-nonylphenol can affect *D. magna* by disrupting ecdysone metabolism (LeBlanc *et al.*, 2000). However, the number of affected genes related to this pathway was too low to be considered significantly enriched by the AmiGO! algorithm. Further research on ecdysone metabolism-related genes in *Daphnia* is thus required to prove or refute this hypothesis.

Common pathways de-regulated by both, SSRIs and 4-nonylphenol, involved enzymes that regulate morphogenesis and tissue differentiation probably involved in molting and reproduction, which are essential processes for growth and energy supply (e.g. cuticle formation, ribosomes, catabolism, transport and binding of essential macromolecules). Similar de-regulated pathways were found to be altered in *D. magna* individuals exposed to narcotic chemicals, pesticides, ammunitions constituents, silver nanoparticles, metals and temperature changes (De Schampheleere *et al.*, 2008; Dom *et al.*, 2012; Garcia-Reyero *et al.*, 2012; Pereira *et al.*, 2010; Vandenbrouck *et al.*, 2011) (Garcia-Reyero *et al.*, 2009; Poynton *et al.*, 2012; Poynton *et al.*, 2007; Poynton *et al.*, 2008; Soetaert *et al.*, 2006; Soetaert *et al.*, 2007). The simplest

explanation to this finding of similar responses to disparate substances is that these pathways are related to general stress responses and not to the observed specific hormetic responses on juvenile developmental rates and female reproduction.

De-regulated metabolic pathways in *D. magna* adults chronically exposed to the studied chemicals were further analysed using KEGG to identify altered metabolic pathways linked to reported effects on energy and carbohydrate metabolism. Both SSRIs and 4-nonylphenol de-regulated similarly key genes such as glyp (glycogen phosphorylase) and upg (UTP:glucose-1-phosphate), both involved in starch and sucrose metabolism, mainly in the transformation of glycogen to glucose (Heckmann *et al.*, 2008). Although both chemical families up-regulated fbp (fructose-1,6-bisphosphatase), only SSRIs down-regulated pfk (phospho-fructo kinase), which is involved in control of glucose homeostasis by allowing to switch from glycolysis to gluconeogenesis (Rider *et al.*, 2004) and is a key gene in the regulation from aerobic to anaerobic metabolism. In a previous study we found that exposure to SSRIs decreases carbohydrate levels in adult *D. magna* females (Campos *et al.*, 2012a), thus down-regulation of pfk may be an adaptive response to maintain minimum levels of glucose and may be linked to reported effects of SSRIs on oxygen consumption rates (Campos *et al.*, 2012a). There are also evidences that serotonin, whose regulation is the pharmacological target of SSRIs, may be involved in the regulation of pfk. In human cells, serotonin increases pfk by binding to the 5-HT(2A) receptor, causing the tyrosine residue of pfk to be phosphorylated via phospholipase C. Because pfk regulates glycolytic flux, serotonin plays a regulatory role in glycolysis (Coelho *et al.*, 2012).

SSRIs and 4-nonylphenol treatments de-regulated acon (aconitase) and ATPCI (ATP citrate lyase), respectively. Additionally, SSRIs specifically up-regulated idh (isocitrate dehydrogenase). Thus SSRIs and 4-nonylphenol de-regulated genes involved in 6 and 4 out of 10 reactions of the Krebs cycle, respectively. These effects could potentially affect oxygen consumption rates and the consumption of carbohydrates to produce energy. SSRIs indeed increased oxygen consumption rates and decreased levels of carbohydrates in *D. magna* (Campos *et al.*, 2012a). 4-nonylphenol is known to increase mRNA

levels of *D. magna* haemoglobin (Ha and Choi, 2009), which may indicate also increasing rates of oxygen consumption.

The beta-oxidation pathway was affected exclusively by SSRIs up-regulating thiolase, which is a key control gene of lipids metabolism and biosynthesis (Liu *et al.*, 2008). 4-nonylphenol and SSRIs up-regulated 2 and 3 genes, respectively, from the sphingolipid biogenesis pathway (cDASE, Glct-1, lace) that are involved in the biogenesis of ceramide, glycosphingolipids and sphingosines. Recently, sphingolipid metabolites, such as ceramide and sphingosine-1-phosphate, have been shown to be important mediators in the signalling cascades involved in apoptosis, proliferation, stress responses, necrosis, inflammation, autophagy, senescence, and differentiation (Hannun and Obeid, 2002).

SSRIs up-regulated specifically dopa-decarboxylase (Ddc) that catalyses the conversion of 5-hydroxytryptophan to serotonin in response to several endogenous or exogenous signals, and has already been shown to be involved in insect cuticle maturation, neuronal regulation, pigmentation patterning and innate immunity (Hodgetts and O'Keefe, 2006). Up-regulation of Ddc in rats exposed to SSRIs has been suggested as a response to the blocking of the re-uptake mechanisms, which leads to low levels of serotonin inside the terminal neuron axon (Choi *et al.*, 2012).

The expression pattern of key genes from significantly enriched pathways such as lipids metabolism (thiolase), Krebs cycle (Acon, idh and ATPCL), tryptophan metabolism (Ddc) and nucleotide metabolism (Faa) was validated by qPCR, confirming the microarray results.

In summary SSRIs and 4-nonylphenol de-regulated different gene pathways in *D. magna* that in most cases could be related to reported phenotypic effects. SSRIs de-regulated more genes than 4-nonylphenol specially in juvenile stages, which support the hypothesis that these compounds act as neuronal disruptors in *Daphnia* (Campos *et al.*, 2012a). SSRIs specifically de-regulated one of their targeted pathways, serotonin metabolism, and key genes from the carbohydrate and Krebs cycle metabolism. 4-nonylphenol specifically targeted genes related to ecdysone, carbohydrate and Krebs cycle in adults. Taken together, the observed changes in the energy-related pathways are consistent with a decrease on reserve carbohydrates and an increase on respiratory metabolism,

in line with our previous observations on the effects of SSRIs in *D. magna* (*Campos et al., 2012a*).

4.5. Acknowledgements

This work was supported by the Spanish MICINN grants (CGL2008-01898/BOS and CTM2011-30471-C02-01) and by the Advance Grant ERC-2012-AdG-320737. Bruno Campos was supported by a fellowship from the MICINN (FPI BES-2009-022741). This work was also partly funded by the US Army Environmental Quality Research Program (including BAA 11-4838). Permission for publishing this information has been granted by the Chief of Engineers.

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CHAPTER 5

Effects of nanoparticles of TiO₂ on food depletion and life-history responses of *Daphnia magna*.

Effects of nanoparticles of TiO₂ on food depletion and life-history responses of *Daphnia magna*.

Bruno Campos¹, Claudia Rivetti¹, Philipp Rosenkranz², José María Navas², Carlos Barata¹

¹Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona, 18-24, 08034 Barcelona, Spain

²INIA, Ctra de la Coruña Km 7.5, E-28040, Madrid, Spain

Published: Aquatic Toxicology 130-134 (2013) 174-183

Abstract

The extent to which different forms of nanoparticles of titanium dioxide (nano-TiO₂) aggregated with microalgae, decreased food levels and hence impaired growth, reproduction and fitness of *Daphnia magna* individuals were studied. Treatments included three different types of nano-TiO₂ differing in their coating or crystalline structure but of similar primary size (20nm) plus a micron-sized bulk material, two exposure levels (1, 10mg/L) and two food ration levels of the microalgae *Chlorella vulgaris* that included a non limiting (1.5µg C/mL) and a limiting one (0.3µg C/mL). Effects were assessed using standardized chronic tests and assays that maximized food depletion in the water column under semi-static and resuspension conditions. Results indicated that the high ion levels in culture medium lead to the aggregation of nanoparticles followed by particle destabilization. Nanoparticle aggregates interacted with the algae cells, forming clusters. Large TiO₂-algae agglomerates settled readily dramatically depleting the concentration of available food for *D. magna*. At limiting food rations food depletion by nanoparticle aggregation had dramatic effects on reproduction and fitness of exposed *D. magna* at 1mg/L irrespectively of the particle form. At high food rations, effects were only observed for one of the nano-TiO₂, P-25, at high exposure levels (10mg/L) under both semi-static and particle re-suspension conditions, which suggest that P-25 effects were mediated by clogging the gut and hence diminishing food acquisition. These results indicate that nano-TiO₂ may affect the transfer of energy throughout the planktonic aquatic food webs increasing the settlement of edible particles from the water column.

5.1. Introduction

The study of the ecotoxicity of manufactured nanomaterials (NM) to aquatic organisms has a relative recent history when compared with that of other toxic substances (Baun *et al.*, 2008a). Several reviews claim that new toxicity procedures should be used to evaluate NM toxicity due to their unique characteristics of size being below 100nm and their inherent toxicity (Baun *et al.*, 2008a; Handy *et al.*, 2012a; Handy *et al.*, 2008a; Handy *et al.*, 2008b). At present, most studies have been focused in determining the bio-availability of NM to aquatic organisms and their direct toxicity to cells, aquatic invertebrates and fish (Baun *et al.*, 2008a). Fewer ones, however, have addressed indirect effects that NM may have in aquatic biota such as in water column invertebrates due to their aggregation properties in aqueous solutions (Li *et al.*, 2011), that can lead to interactions with microscopic (phytoplankton, zooplankton) food particles. There is reported evidence that NM like those of titanium dioxide (nano-TiO₂), fullerenes (C₆₀), or single walled carbon nanotubes (SWCNT) aggregate to microalgae, copepods and *Daphnia* cell surfaces or carapaces, impairing their growth or swimming behaviour (Baun *et al.*, 2008a; Baun *et al.*, 2008b; Li *et al.*, 2011; Metzler *et al.*, 2011; Oberdörster *et al.*, 2006; Templeton *et al.*, 2006). Zooplanktonic grazers such as cladocerans and copepods eat mainly edible particles (i.e. microalgae, bacteria, ciliates) from the water column and hence are very sensitive to changes in food concentration levels. This is especially so in cladoceran species such as *Daphnia magna* whose growth and reproduction is very sensitive to changes in food levels (Barata and Baird, 1998). Therefore, it is crucial to assess to which extent NM can make edible microalgae to sediment faster by forming aggregates with them thereby diminishing food levels for planktonic grazers. Such effects may have profound consequences in the aquatic food web since it will reduce energy transfer from grazers to predators (Baird and Burton, 2001). There are several studies reporting that clay particles of sizes in the low µm range remove microalgae from the water column, a phenomenon that is dependent on the concentration and the size of clays and microalgae and the ionic strength of water (Han and Kim, 2001). NM such as those of nano-TiO₂ may act like clay particles making microalgae to sediment faster (Li *et al.*, 2011). According to Han and Kim (2001)

the closer the clay particle size is to the microalgae size the greater the potential to aggregate with them and make them to settle. There is reported information that nano-TiO₂ aggregates in water reach sizes of few µm in waters with relatively high ionic strength (Sillanpää *et al.*, 2011). Thus it is feasible to hypothesize that no or moderately toxic NM like nano-TiO₂ may affect growth and reproduction of grazers by decreasing food levels. Li *et al.*, (2011) found that nano-TiO₂ and nano-Al₂O₃ decreased the concentration of algae in the water column and consequently impaired growth and reproduction of the cladoceran *Ceriodaphnia dubia*. The biological toxicity of NM is closely related with many physicochemical characteristics such as size, surface area, surface modification, and radical formation. Size is a well known important factor determining nano-TiO₂ toxicity to aquatic organisms since penetration is eased with decreasing particle size and bioavailability is increased, so that more particles can be deposited inside the cell (Kim *et al.*, 2010; Lovern and Klaper, 2006; Metzler *et al.*, 2011). The phase composition of nanoscale titanium particles is also known to affect toxicity under UV light with anatase TiO₂ being 100 times more toxic to human fibroblast or lung epithelial cells than an equivalent sample of rutile (Sayes *et al.*, 2006). There is, however, few if any study that have compared the aquatic toxicity of nano-TiO₂ having different phase composition to aquatic species. It is difficult, however, to distinguish experimentally if effects of NM on growth and reproduction are due to direct toxicity, to a lower availability of algae as food, to decreased food intake due to ingestion of NM that are not edible or due to the contribution of all factors. Nevertheless, there is still substantial controversy on the mode of toxic action of NM to aquatic organisms due to problems, for example, to establish exposure pathways (Handy *et al.*, 2012b). Most nanotoxicity studies followed conventional protocols designed to test dissolved chemical species that may not be totally applicable to nanoparticles (Handy *et al.*, 2012b). For example in the semi-static testing conditions of the Organization for Economic Cooperation and Development protocols (OECD, 1981), NM are likely to aggregate with themselves and with algae, increasing algae sedimentation rates and hence diminishing food levels for grazers such as *D. magna* that eat algae from the water column. In relation to this, test vessel volumes usually used to conduct chronic tests with cladoceran species are quite small (30–150ml), having small

water columns (3–6cm) and hence may also minimize algae sedimentation effects. The above mentioned limitations, however, can be avoided by performing tests with larger vessels holding larger water columns under semi-static and continue water column resuspension conditions. Here we aim to evaluate the extent to which different forms of nano-TiO₂ aggregate with microalgae, decreasing food levels and consequently growth and reproduction of *D. magna* individuals. To accomplish our objective we performed different experiments using three different types of nano-TiO₂ differing in their coating or crystalline structure but of similar primary size (20nm) plus a micron-sized bulk material, two nano-TiO₂ exposure levels (1, 10mg/L) and different food ration levels of the microalgae *Chlorella vulgaris* that included non limiting (1.5µg C/mL) and limiting food conditions (<1µg C/mL) (Barata and Baird, 1998). Effects were assessed using three different types of assays. Assays included chronic reproduction 21-day tests using semi-static conditions that followed OECD protocols (OECD, 1981) and tests performed in 1L bottles with a water column height of 16cm. The latter included two exposure scenarios: semi-static and re-suspension of algae. Particle size and aggregation measurements of nano-TiO₂ were conducted using scanning and transmission electron microscopy (SEM and TEM, respectively), dynamic light scattering (DLS) and particle counter procedures. Algae concentration measurements were based on chlorophyll a and effects on *D. magna* were determined from growth and reproduction performance measurements.

5.2. Material and methods

5.2.1. Nanoparticles

Two of the used nano-TiO₂ has been supplied by the repository of the European Commission (EC) Joint Research Centre at Ispra, Italy. In order to facilitate the collection of data and the comparison with other studies performed with same nano-TiO₂, the codes used in this work are those of the repository. NM-103 and NM-104 are TiO₂ of rutile modification, with a TiO₂ total content of 89%, a primary crystal size according to Scherrer of 20nm (the Scherrer formula is used in X-ray diffraction to correlate the size of solid nanoparticles with the broadening of a peak in a diffraction pattern, (Scherrer, 1918) and a specific

surface area according to BET of $60\text{m}^2/\text{g}$ (Brunauer–Emmet–Teller method, (Brunauer *et al.*, 1938). According to the received information NM-103 exhibits hydrophobic properties while NM-104 is hydrophilic. Both nanomaterials are used as UV screening agents in sunscreen products and NM- 104 has received an organic treatment with glycerin. The other NM used in this work was P-25 (Evonik Degussa, Germany). It is a TiO_2 of anatase–rutile modification (78% anatase, 14% rutile and 8% amorphous phase, according to (Ohtani *et al.*, 2010)). The manufacturer reports 99.5% purity and a specific surface area according to BET of $50\pm15\text{m}^2/\text{g}$. A primary size of 21nm has been reported (Sigma–Aldrich, <http://www.sigmaaldrich.com/>). Finally a micro TiO_2 was used as reference: Tiona AT-1 (Cristal Global, MD). The manufacturer reports that the crystal form is anatase, with 98.5% TiO_2 content and with untreated surface. Initial stock solutions of particle suspension of 1 g/l were dispersed with the aid of an ultrasonic bath during 30minutes in MilliQ water and left for 24h in agitation in darkness prior to be used to prepare test solutions.

5.2.2. Particle characterization

5.2.2.1. Pristine particles

Properties of particles were characterized for primary particle size and aggregates (or secondary particle size distribution). TEM pictures of the different particles were taken. Particle samples were prepared by ultrasonication (5–10min) with an ultrasonic bath Elmasonic S40 (Elma, Germany) at maximum energy output to eliminate aggregates and ensuring the possibility to image single particles. A few drops of suspension were allowed to evaporate in a copper grid at room temperature and examined under a JEOL transmission electron microscope, model 2000FX (JEOL, Ltd., Japan) operating at 200 kV.

5.2.2.2. Particles in suspension

Particles were dispersed in ASTM hard synthetic water, pH 7.8 (ASTM, 1980) following the above mentioned methods and left to settle for 24h, then the aggregate size of nanoparticles in the water column was determined with dynamic light scattering (DLS) using a Zeta-Sizer Nano ZS (Malvern

Instruments Ltd., Malvern, UK). ASTM hard water without particles was used as a control. Before measuring the samples the instrument temperature was set at 20°C. Three independent measurements were taken with 4 readings per measurement, each reading consisting of six runs of 10s duration. The mean hydrodynamic size was calculated from the histogram of size distribution frequency for each nanoparticle. The polydispersity index (Pdi) that informs about the width of the size distribution was also included. Nanoparticle concentrations in water were also measured indirectly using absorbance measurements at 348nm calibrated against a standard of 8 points.

5.2.3. Culture conditions

A single clone F of *D. magna*, which has been the subject of many investigations (Barata and Baird, 2000), was used. Individual cultures of 10 animals/L were maintained in ASTM hard synthetic water with the addition of a standard organic extract (Baird *et al.*, 1989a). Individual cultures were fed daily with *C. vulgaris* Beijerinck (5×10^5 cells/mL). Algae were cultured in Woods Hole MBL medium (Nichols, 1973) with constant aeration, collected in the exponential phase and re-suspended in ASTM hard water (ASTM, 1980). Algal cell concentration was calculated using a particle counter Coulter Multisizer (Beckman Coulter, CA). The culture medium was changed every other day, and neonates were removed within 24 h. Photoperiod was set to 14h light: 10h dark cycle and temperature at $20 \pm 1^\circ\text{C}$. Light was provided with neon fluorescent lamps that according to the manufacturer have low UVA emissions ($< 10 \mu\text{W/cm}^2$), which are roughly 300-fold lower than the typical maximum (clear-sky, mid-day, mid-summer) intensity in temperate regions such as Duluth (USA) and Paris (France) (Ma *et al.*, 2012).

5.2.4. Experimental design

5.2.4.1. Algae assays

Effects of the four studied particle suspensions (NM-103, NM- 104, P25, Tiona) on the formation of particle–algae aggregates and its effect on algae sedimentation rates were tested at two concentrations (1 and 10mg/L) and at

low and high food ration levels of *C. vulgaris* (1×10^5 and 5×10^5 cells/mL) with an equivalent carbon content of 0.3 and $1.5\mu\text{g}$ C/mL, respectively, that correspond to limiting and non-limiting food levels to *D. magna*, respectively (Barata and Baird, 1998). To determine carbon content, three replicate algae samples of known volume and concentration were freeze dried, weighed on a microbalance and their C:N:H content measured in a Thermo Electron Flash 1112 elemental analyser (Thermo Scientific, UK). Particle test suspensions were prepared from appropriated dilutions of stocks and were dispersed with ultrasonication for 30min in ASTM hard water and kept in continuous agitation. Experiments were conducted in 1.2L bottles filled with 1L of ASTM with the required algae and particle suspensions and then left to settle for 24h prior to sampling. Algae concentrations in those treatments with nanoparticles cannot be assessed using particle counter methods due to particle reading interference and hence were determined from chlorophyll a measurements. Water samples (100mL) were carefully taken from the mid-point of the water column (approximately 7.5cm from the bottom) with the aid of a glass pipette. Water samples were first filtered using fibre-glass filters (Whatman GF/F $0.2\mu\text{m}$, Whatman, UK) and then chlorophyll was extracted with acetone overnight following established methods (USEPA, 1997). Extracts were then measured at 664, 647, 630 and 750nm by spectrometry (DU720 Beckman Coulter, CA). The concentration of chlorophyll a was calculated using the trichromatic equations of Jeffrey and Humphrey (1975) and converted to cell counts using a calibration curve of 8 data points. Final results were then expressed in μC of algae/mL. Measurement of the size of algae aggregates with nanoparticles were visualized with the aid of a Hitachi S-3500N (Hitachi High Technologies Corporation, Japan) scanning electron microscope (SEM) and measured using a particle counter Coulter Multisizer (Beckman Coulter, CA) in three independent experiments. To visualize algae aggregated with particles in the SEM, 1–5mL of algae sample was filtered in a Nucleopore filter (Whatman), $0.8\mu\text{m}$ pore size, and fixed in glutaraldehyde at 3% prepared in ASTM hard water during 1h. After that the sample was washed for 20min in ASTM and dehydrated using ethanol serial dilutions. Filters were air dried, glued to SEM stubs with colloidal silver and sputter-coated with gold–palladium. The samples were examined with a Hitachi S-3500N SEM operating at 5kV. To measure the metallic composite of the aggregates an X-ray

microanalysis, Quantax 400 energy dispersive spectrometer Si (Li) detector (Bruker AXS, Germany), was used attached to the SEM and working at 15kV.

5.2.4.2. *D. magna* reproduction tests

Effects of the four studied particle suspensions (NM-103, NM-104, P-25, Tiona) on *D. magna* growth and reproduction were tested at two concentrations (1 and 10mg/L) and at low and high food ration levels of *C. vulgaris* (0.3 and 1.5 μ g C/mL, respectively). Particle test suspensions were prepared similar than in the algae assays. Four different assays were performed:

Experiment 1: included a 21-day reproduction test following established OECD (1981) protocols. Neonates (<24 h old) were exposed individually until their fourth brood (approximately 21–23 days at 20°C) to the studied particle concentrations across low and high food ration levels in 100ml of ASTM hard water, in 120mL screw top glass jars (6cm water column), with the addition of a standard organic extract (Barata and Baird, 1998). Exposure solutions with algae were changed every day and were replicated ten times. In experiments 2 and 3, neonates (<24h old) were exposed until their first brood (approximately 9–11 days at 20°C) to the studied particle concentrations and algae treatments (low and high food ration levels) in groups of 10 individuals cultured in 1L of ASTM hard water, in 1.2L screw top glass bottles holding a water column of 16 cm, also with the addition of a standard organic extract (Baird *et al.*, 1989b).

Experiment 2: cultures were maintained like in the experiment 1 in semi-static conditions. The medium was renewed every 24h.

Experiment 3: cultures were maintained in a rotatory wheel at 2rpm to allow continuous re-suspension of algae and particles until medium replacement every 24h.

Experiment 4 was performed like experiment 2 but with algae alone that was dosed at food ration levels matching the food levels not removed by sedimentation with the studied nano-TiO₂ at 1 and 10mg/L. These food ration regimes correspond roughly to half and 1/5 of the levels initially used at high food (0.75, 0.3 μ g C/ml, respectively) and to half and 1/4 the levels initially used at low food (0.15, 0.075 μ g C/ml, respectively). The high food ration level used in experiments 1–3 (1.5 μ g C/mL) was also included as a reference treatment.

Measured responses included individual survival, age, body size and clutch size at first reproduction, cumulative reproduction (only for experiment 1) and population growth rates. Body length was taken from the head to the base of the spine using the ImageJ software (<http://rsb.info.nih.gov/ij/>) and a Nikon stereo-scopic microscope (SMZ 150, Nikon, Spain). Population growth rates were determined as the intrinsic rate of increase (r), which was computed iteratively from the Lotka equation (Eq. 5.1) using the measured age, specific survival and fecundity rates:

$$\sum_{x=0}^{\infty} e^{-rx} l_x m_x = 1 \quad (\text{eq. 5.1})$$

where l_x is the proportion of the females surviving to age x (days) which was set to 1 since no mortality occurred during assays; m_x is the number of juveniles produced per surviving female between the ages x and $x+1$. The age at birth was set to 0. The 95% confidence intervals were estimated by the jackknife method (Meyer *et al.*, 1986).

5.2.5. Data analyses

Due to experimental constraints it was not feasible to perform all experiments at once, neither was it possible to test particle suspension effects at both food levels simultaneously. Accordingly, each experiment and food ration was analyzed separately by one way ANOVA to exclude confounding effects of inter-trial/experiment variation (Barata and Baird, 1998). When significant ($P<0.05$) treatment effects occurred, post-hoc Tukey's or Dunnet's tests were performed to compare treatments. Prior to analyses all data except age at first reproduction were log transformed to improve normality and variance homoscedasticity (Zar, 1999). Effects of treatment on age at first reproduction were analyzed by non-parametric Kruskal–Wallis test. Analyses of r were based on jackknife pseudovalues (Meyer *et al.*, 1986). Tests were conducted with the aid of the statistical package IBM SPSS v19.

5.3. Results

5.3.1. Particle characterization

Primary particle size determined by transmission electron microscope (TEM) confirmed the sizes reported by manufacturers (i.e. approximately 20nm for NM-103, NM-104, P-25 and approximately of 200nm for Tiona). DLS measurements are reported in Table 5.1 and indicate in most cases aggregate sizes greater than 1µm and bigger at 10mg/L than at 1mg/L. Polydispersity indexes were also higher than 0.5 in 6 out of 8 particles*dose combinations, which denoted the existence of polydispersity.

After 24h nanoparticle concentration level estimates decreased from 6.5% to 39% of nominal values at 1mg/L and about 70% at 10mg/L (Table 5.1).

Table 5.1. Nano-TiO₂ and micron sized particle characterization by dinamic light scattering (DLS) and particle concentration estimated from absorbance measurements at 348nm. Pdi, Polydispersity index. Results are depicted as mean±SD

	NM103			NM104		P-25		Tiona		
	1 mg/L	N	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Hydrodynamic size (nm)	12	255.1	42.9		312.1	26.0	412.8	167.5	722.6	518.4
Pdi	12	0.36	0.03		0.80	0.04	0.89	0.14	0.98	0.28
Concentration 24 h (mg/l)	4	0.81	0.08		0.93	0.1	0.8	0.06	0.61	0.06
<hr/>										
10 mg/L										
Hydrodynamic size(nm)	12	1076.6	33.4		699.0	9.8	1354.9	35.8	1502.2	61.9
Pdi	12	0.66	0.03		0.91	0.05	0.45	0.01	0.63	0.02
Concentration 24 h (mg/l)	4	2.80	0.30		3.12	0.21	2.70	0.28	2.33	0.31

5.3.2. Algae–particle aggregates

The tested nano-TiO₂ and the micron-sized reference material aggregated to algae as shown in Figure 5.1-5.4. A SEM image of those aggregates is depicted in Figure 5.1 showing that at high food rations, aggregates to algae were larger at 10mg/L than at 1mg/L. Measurement of the metallic composition of aggregates by X-ray microanalysis confirmed that they were of titanium. The size of algae–particle aggregates determined by means of a Coulter Multisizer showed that in most cases nano- and micron-sized TiO₂ formed significantly (P<0.05, based on ANOVA) larger aggregates with algae than algae alone (Figure 5.2).

The hydrophobic nano-TiO₂ NM-103 formed larger aggregates with algae cells at high food ration conditions and high exposure levels (10mg/L, Figure

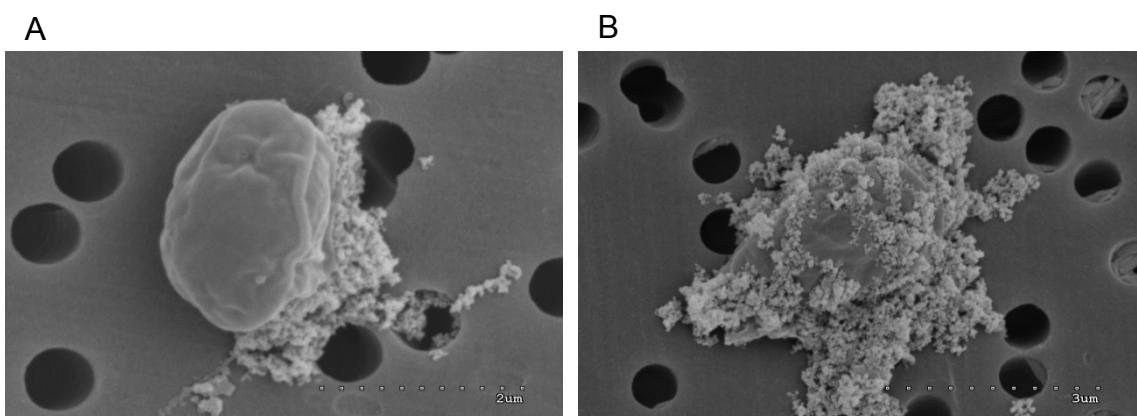


Figure 5.1. SEM Images of algae cells in the presence of 1 mg/L (A) and 10 mg/L (B) of P25- TiO_2 at high food levels.

5.2D), whereas P-25 did so at low food levels and low exposure suspensions (Figure 5.2A). The micron-sized Tiona formed the smallest aggregates with algae. Nevertheless it is important to note that such aggregate measurements are likely to be under-estimate by the Coulter Multisizer since it counts and measures particles assuming a spherical shape, which was not the case as shown in Figure 5.1. Further information on the frequency distribution of algae–particle aggregates in volumetric % is in Figure 5.3.

Algae sedimentation results expressed as food levels ($\mu\text{g C/ml}$ of the algae *C. vulgaris*) remaining in the water column after 24h are depicted in Figure 5.4. Again the results obtained evidenced that the tested TiO_2 –particle suspensions decreased significantly ($P<0.05$, based on one way ANOVA) algae concentrations in water at low and high food ration levels. Such effects were greater at 10mg/L than at 1mg/L. Nano- TiO_2 suspensions of 1mg/L decreased algae levels about 2-fold while those at 10mg/L did so 4 and 9-fold, depending on the food ration level. Effects on depletion of algae concentration levels were greater under exposure to the tested nanoparticles than to the micron-sized reference material Tiona. There were also significant ($P<0.05$) differences among the tested nanoparticles with the hydrophilic coated one (NM-104) and P-25 having the greatest effects. Within each food level Pearson correlation coefficients of particle–algae aggregate size and sedimentation rates were negative and significant ($P<0.05$; $N=9$; -0.71 and -0.64 at low and high food levels, respectively), which means that the larger the algae–particle aggregate the lower the food level remaining in the water column (for further information, those relationships are depicted in Figure 5.5).

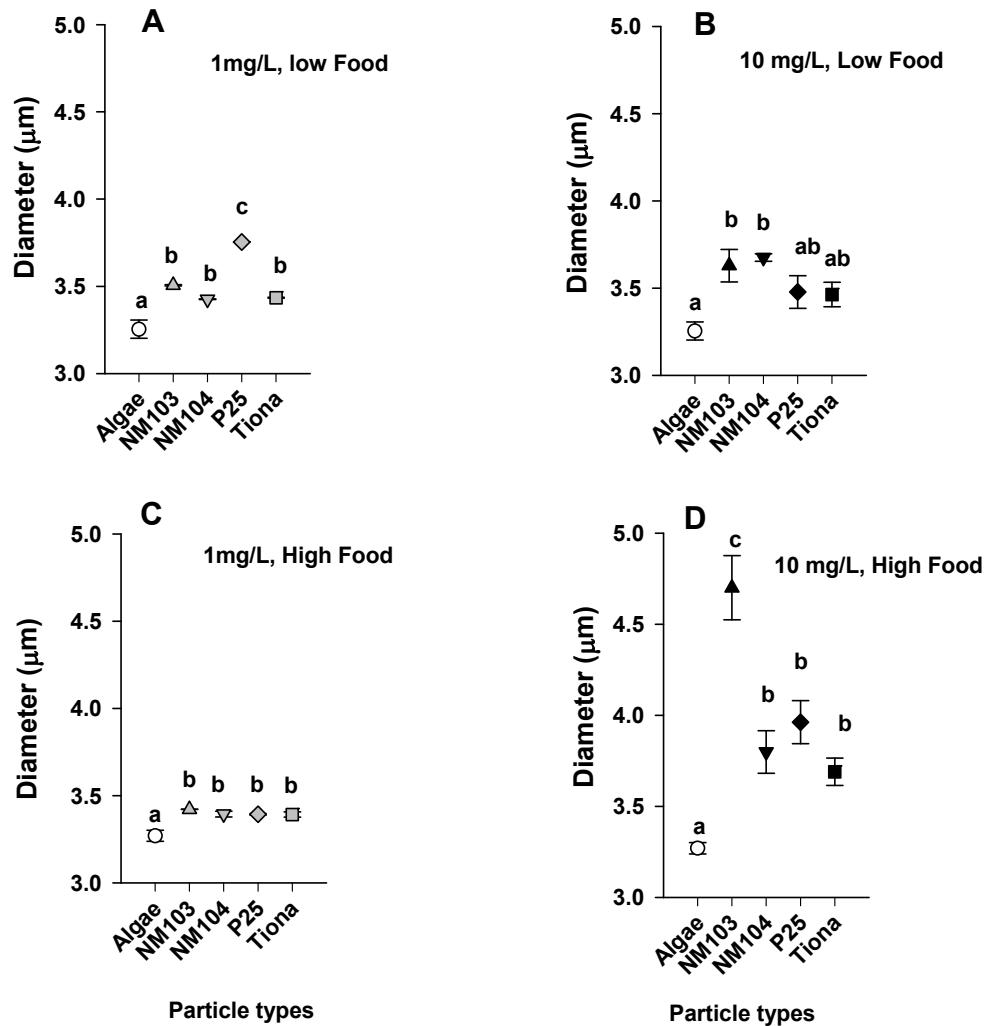


Figure 5.2 Mean algae and particle-algae aggregate sizes of the studied nano and micron-sized TiO₂ particles at low (A, B) and high (C, D) food ration levels. White, grey and black symbols correspond to 0, 1 and 10 mg/L of particle suspension, respectively. Within each food level and particle suspension concentration different letters indicate significant ($P < 0.05$) differences following ANOVA and Tukey's test. Each assay was replicated three times. Errors are SE.

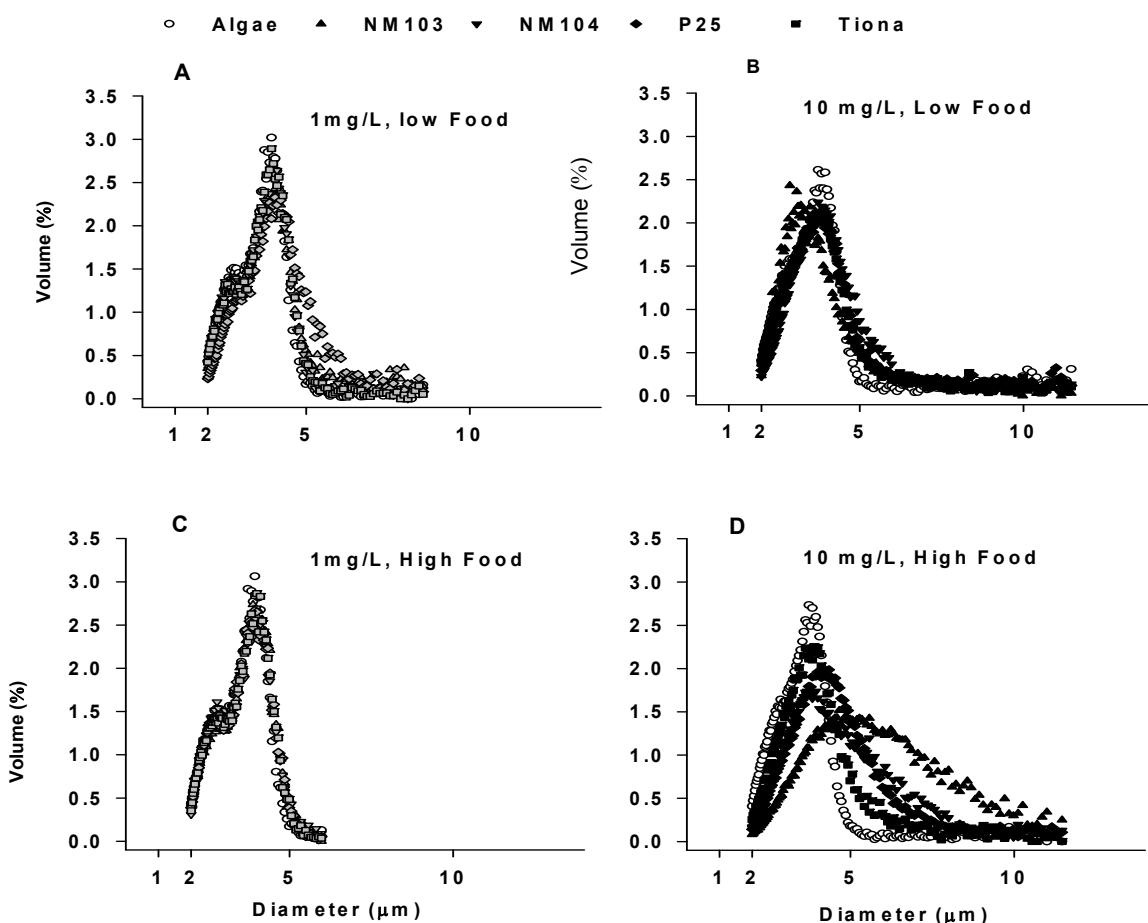


Figure 5.3. Algae and particle-algae size distributions of the studied nano and micron-sized TiO_2 particles dosed at 1 mg/l and 10 mg/l at low (A, B) and high (C, D) food ration levels. White, grey and black symbols correspond to 0, 1 and 10 mg/L of particle suspension, respectively.

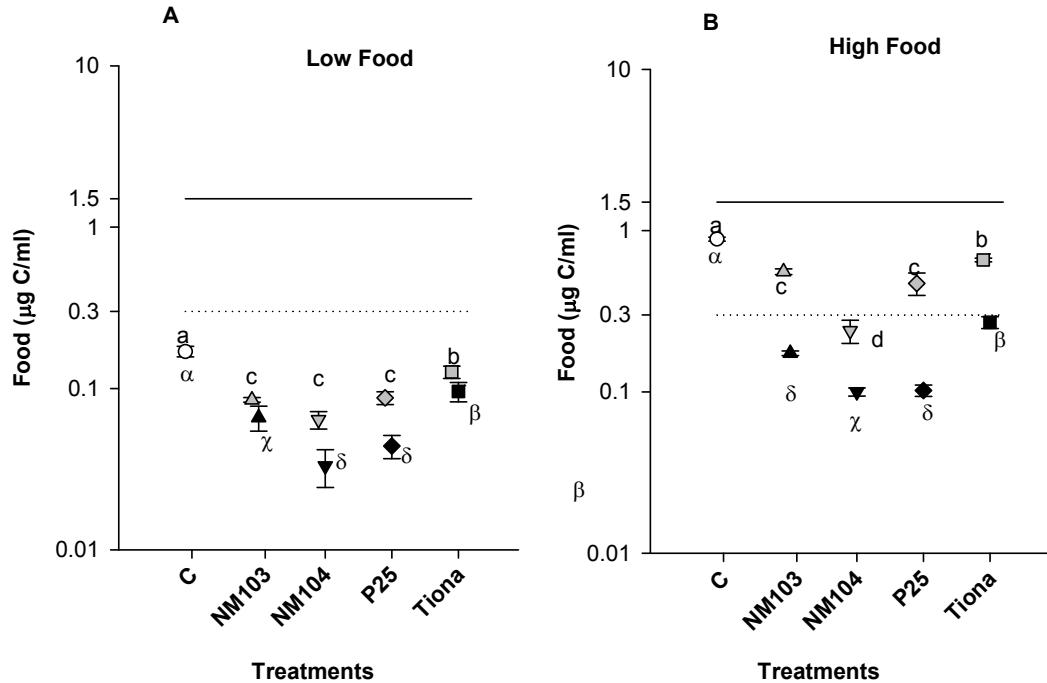


Figure 5.4 Algae concentrations measured as $\mu\text{g C}/\text{mL}$ remaining in the test medium after 24h across the tested particle suspensions at low (A) and high (B) food ration levels. Straight and dotted lines indicate non-limiting and limiting food levels, respectively, a and the location of the initial algae concentration levels. White, grey and black symbols correspond to 0, 1 and 10mg/L of particle suspension, respectively. Within each particle suspension level different Arabic (1mg/L) or Greek (10mg/L) letters indicate significant differences following ANOVA and Tukey's test. Each experiment was replicated three times. Errors are SE.

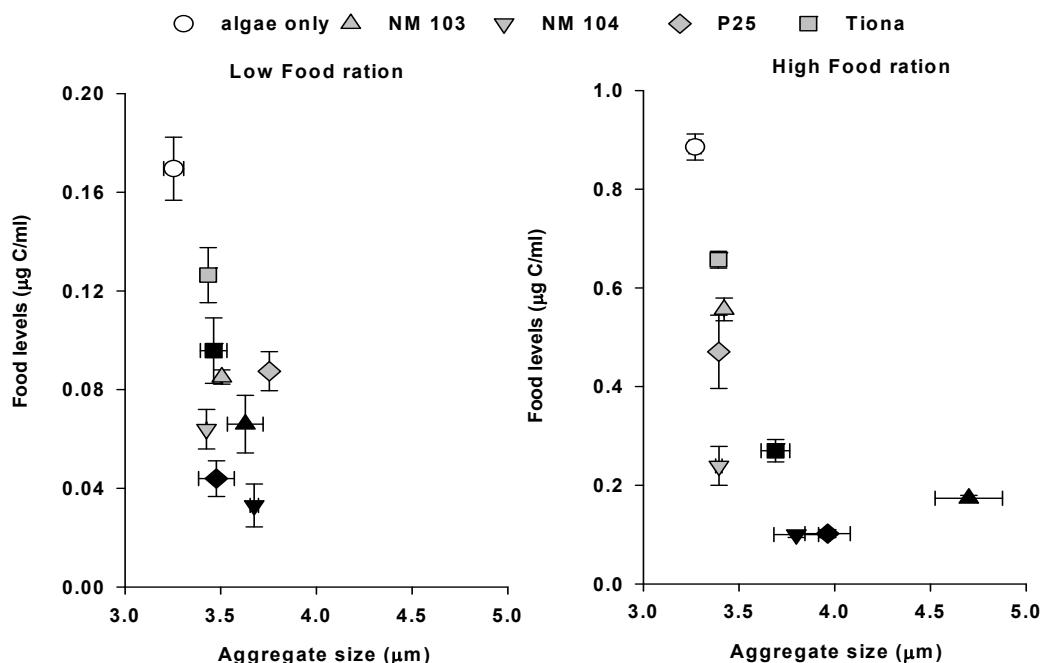


Figure 5.5. Relationships between algae-particle aggregate size and algae sedimentation rates (expressed as concentration of algae remaining in the water column after 24h) at low and high food ration levels. Error bars are SE ($N=9$). White, grey and black symbols correspond to algae only, 1 and 10mg/l of particle suspensions, respectively.

Table 5.2. ANOVA results on *D. magna* traits for the three assays performed with the four particle suspensions (NM103, NM104, P-25, Tiona) dosed at two concentrations (1, 10mg/L) across two food ration levels (low and high). Results from a forth experiment comparing *D. magna* life-history performance across five different ration levels are also included. Only degrees of freedom (df), Fisher's coefficient (F), Chi-square (χ^2) and significant levels are depicted. ns P \geq 0.05; *0.01<P<0.05, **P<0.01. Traits are: age at first reproduction (Age), clutch size at first reproduction (Fec 1st), body length at maturity (Size mat) fitness (r),body length at the end of 21 d test (Size end), and total fecundity in 21 d tests (Fec Tot).

	Ag		Fec 1st		Size mat		r		Size end		Fec Tot	
	df	χ^2	df	F	df	F	df	F	df	F	df	F
1. Chronic Exp												
High	8	2.3	8,6	3.6**	8,6	5.8**	8,7	2.2*	8,5	1.7	8,6	4.0**
Low	8	23.8*	8,6	4.9**	8,6	2.8**	8,7	6.3**	8,6	6.0*	8,6	11.4*
Designed experiments												
2.Semi-static Low	8	19.6*	8,6	12.5**	8,6	4.6**	8,6	12.8**				
2.Semi-static High	8	25.8*	8,6	4.4**	8,6	2.7*	8,6	19.9**				
3.Re-suspension	8	9.30	8,7	7.9**	8,7	1.4	8,7	10.0**				
3.Re-suspension	8	24.5*	8,6	4.8**	8,6	1.7	8,6	14.9**				
4. Ration Food level	4	50.6*	4,5	104.9*	4,5	83.2*	4,5	180.9*				

5.3.3. *D. magna* responses

In the conventional chronic reproduction test the tested particle suspension affected significantly (P<0.05) most of the studied traits (Table 5.2). Post-hoc Dunnet's multiple comparison tests, however, indicated that most detrimental effects relative to control treatments occurred at low food levels in animals exposed to NM-103 and P-25 at 10 mg/L (Figure 5.6). Significant (P < 0.05) effects on body length were observed for NM-103, NM-104 and P-25: at first clutch females exposed to 1 and 10mg/l of P-25 at high food ration levels were bigger and smaller than controls, respectively; at low food ration levels the size of females at first clutch exposed to 10mg/l of MN-103 and that of 21-day old females exposed to 1mg/l MN-104 and to 10mg/l P-25 were bigger than controls (Figure 5.6). Effects on population growth rates (r), which is a fitness measurement that integrates effects on survival, developmental and reproduction rates denoted significant effects of NM-103 only at 10mg/L at low food ration levels. The use of new experimental designs allowed an increase in the sensitivity of observed effects of the studied nanoparticles. In the semi-static test (Figure. 5.7, left panel of graphs) where animals were exposed in bottles of 1L, significant (P<0.05) detrimental effects on age at first reproduction, clutch size, body length and r were observed at high food ration levels and 10mg/L of P-25. At low food ration levels significant (P<0.05) detrimental effects relative

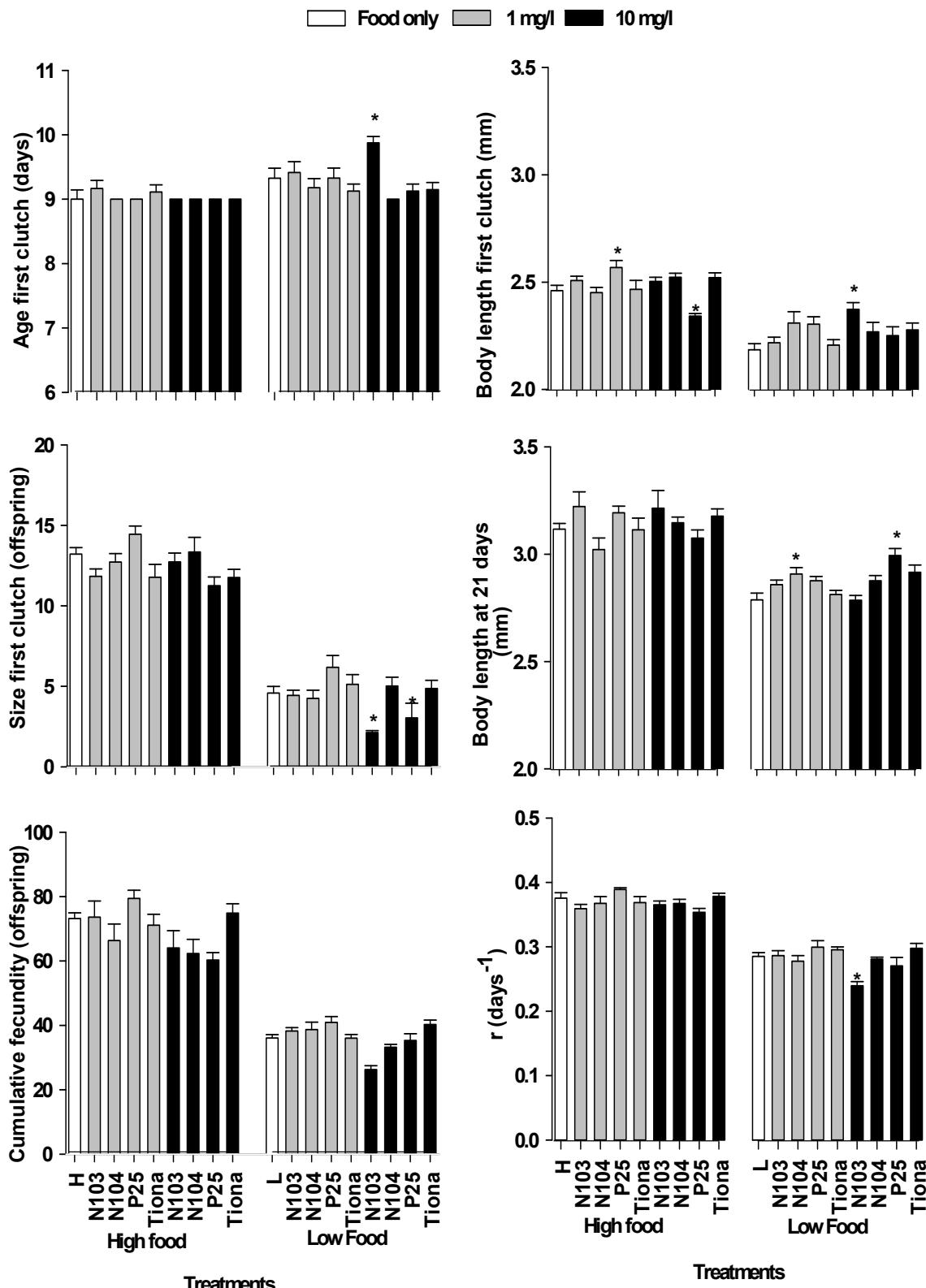


Figure 5.6. Life-history responses of *D. magna* individuals exposed to the studied particle suspensions at low (L) and high (H) food ration levels in a 21 d chronic test. White, grey and black bars correspond to 0, 1 and 10 mg/L of particle suspension, respectively. Within each food level * indicates significant ($P < 0.05$) differences from control treatments following ANOVA and Dunnett's test. Errors are SE ($N=10$).

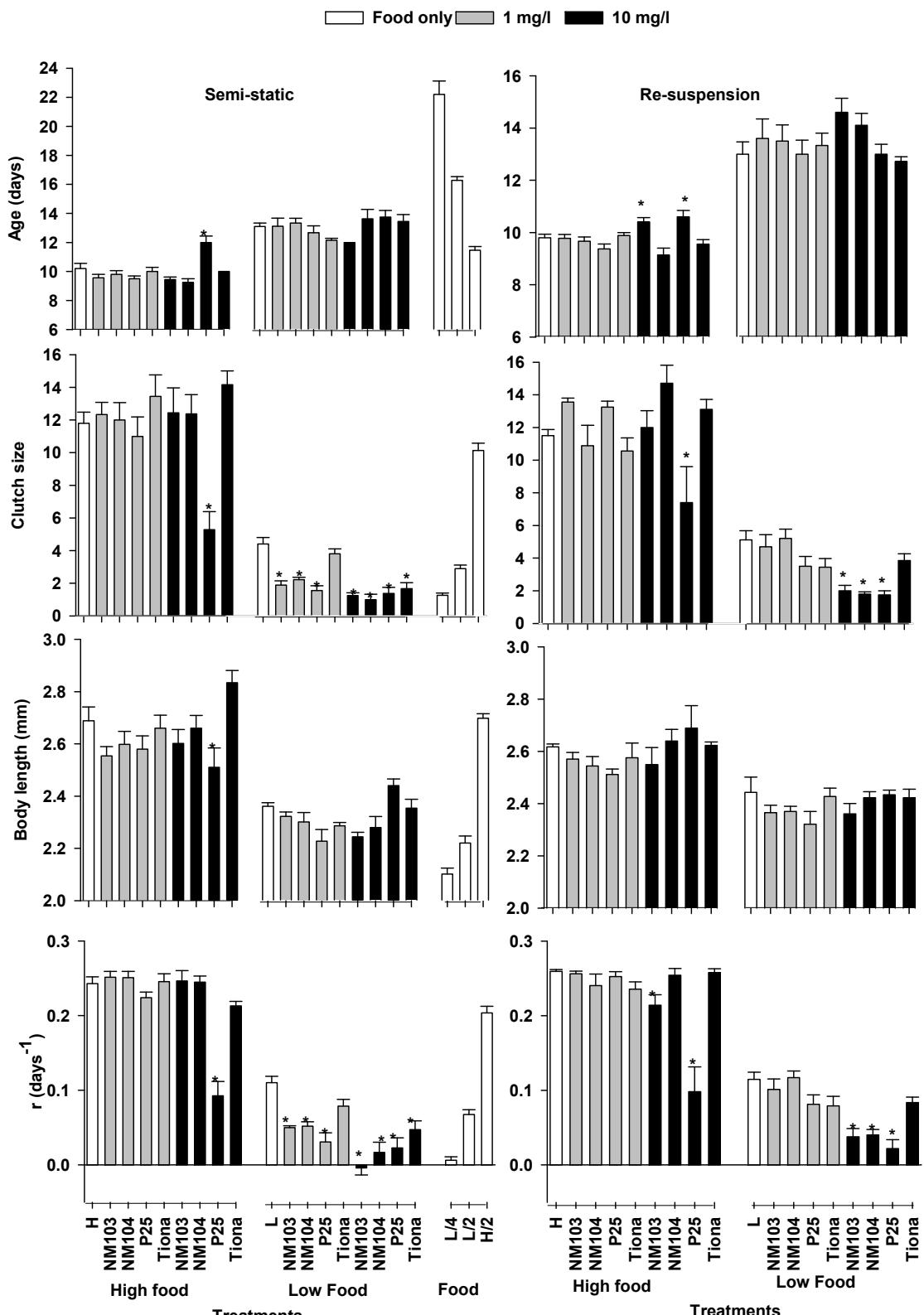


Figure 5.7. Life-history responses of *D. magna* individuals exposed to the studied particle suspensions at low (L) and high (H) food ration levels in 1L medium under semi-static and re-suspension conditions. Results from the food ration experiment dosed at levels two fold lower than high food (H/2) and two and fourth fold lower than low food (L/2, L/4) are also depicted. For clarity in the food ration experiment results for H and L treatments are not included but are depicted in Table 5.4. White, grey and black symbols correspond to 0, 1 and 10mg/L of particle suspension, respectively. * indicates significant ($P<0.05$) differences of particle treatments from food only control treatments following ANOVA and Dunnett's test. Errors are SE ($N=10$).

to controls on clutch size and r were observed even at 1mg/L in all the tested nano-TiO₂ (Figure. 5.7). Effects of Tiona occurred only at low food ration levels and high exposure levels (10 mg/L) (Figure. 5.7). Comparison of observed life-history effects of *D. magna* individuals exposed to the studied particle suspensions with those obtained at food ration levels matching what was not removed by sedimentation showed the following: in the experiment performed at high food ration levels, observed fitness (r) effects at 10mg/L of P-25 were not significantly ($P<0.05$ based on ANOVA tests) different from those obtained at L/2 (Figure 5.7), which roughly corresponds to food depletion values measured in Figure 5.4B. In the experiment performed at low food ration level, life-history effects observed at 1 and 10mg/L of nano-TiO₂ suspensions were similar to effects measured at food levels matching those not sedimented according to Figure 5.4A (L/2, L/4, Figure 5.7). The re-suspension experiment conducted under similar conditions as the semi-static test but allowing particles and algae to re-suspend continuously provided similar results at high food ration levels but effects at low food ration ones were only observed at 10 mg/L (Figure 5.7 right panel graphs).

Table 5.3. Pearson correlation coefficients between particle-algae aggregation size, sedimentation rates measured as algae concentration levels remaining in the water column after 24h and r -values in the chronic, semi-static and re-suspension assays at low and high food ration levels. * $0.01 < P < 0.05$; ** $0.01 < P < 0.01$, N=10

	r-chronic	r-semi-static	r –re-suspension
Low Food			
Aggregate size	-0.13	-0.77*	-0.44
Sedimentation	0.40	0.87**	0.68*
High food			
Aggregate size	-0.36	-0.22	-0.80**
Sedimentation	0.35	0.39	0.52

Correlation analyses were restricted to population growth rate since it is a better measure of fitness than the other studied traits. Interestingly, at low food ration levels population growth rates were correlated significantly ($P<0.05$; N=10) with particle–algae aggregate size and with algae concentration levels remaining after 24h (i.e. sedimentation rates) in the semi-static test, with sedi-

Table 5.4. Life-history responses of *D. magna* cultured at different food ration levels matching those remaining in the water column at 24 h following nanoparticle treatments. High and low food ration levels have also been included for comparison purposes

Food ration µgC/ml	N	Age (days)		Clutch (number)		Length (mm)		r (days ⁻¹)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
L/4	10	22.21	0.92	1.26	0.13	2.1	0.02	0.006	0.005
L/2	10	16.14	0.26	2.89	0.22	2.22	0.03	0.065	0.006
L	10	13.1	0.16	4.4	0.63	2.36	0.03	0.11	0.013
H/2	10	11.46	0.26	10.13	0.45	2.7	0.02	0.204	0.009
H	10	10.2	0	11.8	0.38	2.69	0.02	0.243	0.004

mentation rates in the re-suspension test and with none of these variables in the chronic test (Table 5.3). At high food levels population growth rates were correlated significantly ($P < 0.05$; $N = 10$) with particle–algae aggregate size in the re-suspension test (Table 5.3). Further information on the relationships between fitness (r responses) and algae–particle aggregate size or sedimentation rates are depicted in Figure 5.8.

5.4. Discussion

The particle size distribution determined by laser scattering analysis showed that nano-TiO₂ was consistently aggregated in test suspensions (1 and 10mg/L). The agglomerate size and polydispersity index were also concentration-dependent with the higher TiO₂ concentrations showing greater agglomerate size. Particle–TiO₂ concentration estimates using absorbance measurements showed that by the end of 24h, particle sedimentation caused loss of particle–TiO₂ suspensions of 21 and 72% from initial levels of 1 and 10mg/L, respectively. The observed aggregation effects across nano-TiO₂ concentrations are in agreement with other descriptions of nano-TiO₂ behaviour in high ionic strength media (Sillanpää *et al.*, 2011). Therefore, the culture medium with high ion levels leads to the aggregation of nanoparticles followed by particle destabilization. Nanoparticle aggregates interacted with the algae cells, forming clusters as shown in Figures 5.1, 5.2, 5.3. Large TiO₂ –algae agglomerates settled readily dramatically depleting the concentration of food for *D. magna*. Results of Figure 5.4 indicated that at low food ration levels, algae food concentrations were reduced by about 2 and 4-fold in the presence of 1

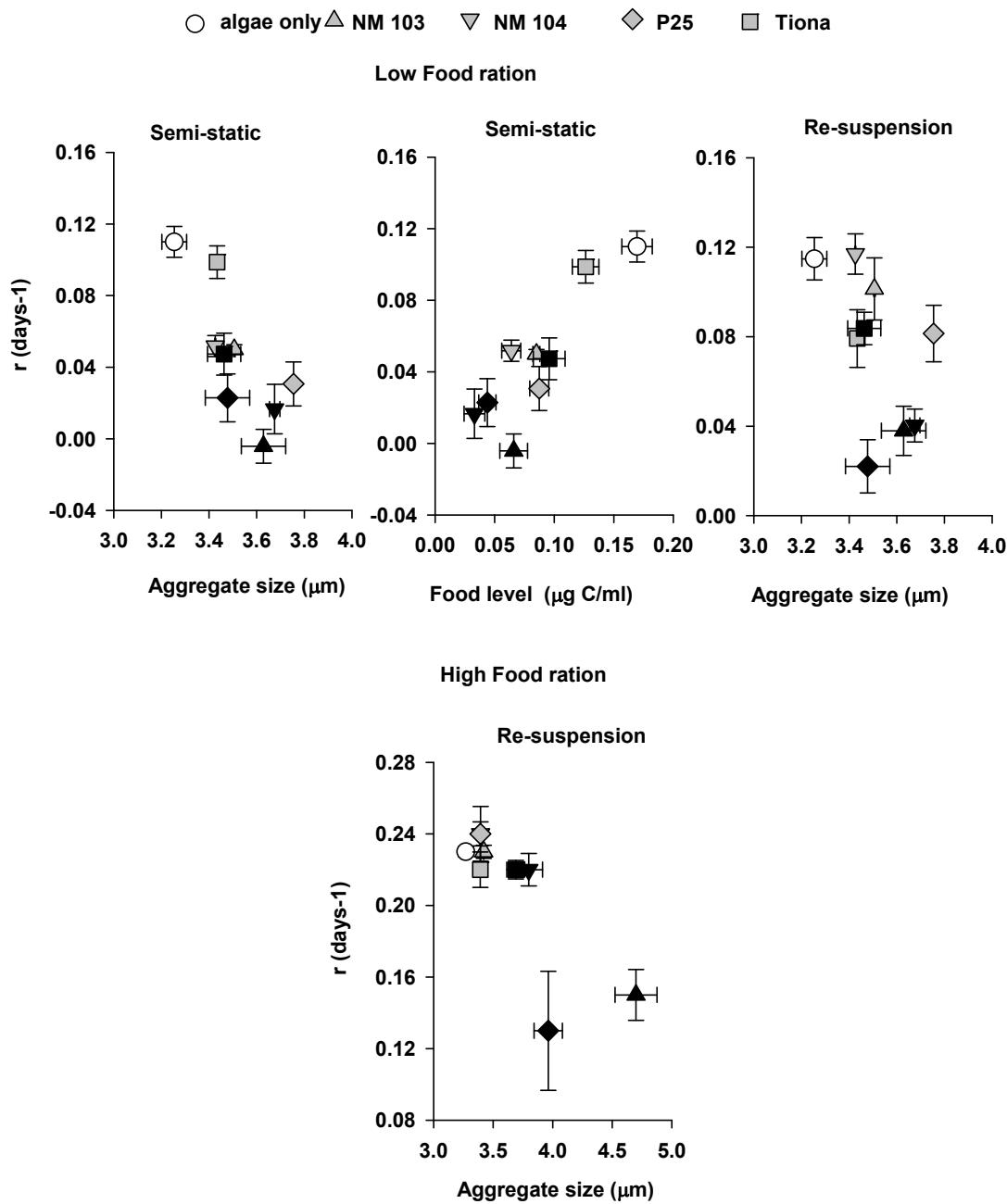


Figure 5.8. Significant ($P < 0.05$, Table 5.3) relationships between algae-particle aggregate size or algae sedimentation rates (expressed as concentration of algae remaining in the water column after 24 h) and fitness (r -responses) across food ration levels and experiments. Error bars are SE ($N = 9$). White, grey and black symbols corresponds to algae only, 1 and 10 mg/L of particle suspensions, respectively.

and 10mg/L of nano-TiO₂, respectively. At high food ration levels food reductions by nanoparticle suspensions were greater being about 2 and 9-fold for 1 and 10mg/L of nano-TiO₂, respectively. These results agree with reported aggregation effects of nano-TiO₂ in algal cells and subsequent algae sedimentation (Li *et al.*, 2011; Metzler *et al.*, 2011).

Life-history theory and empirical data indicate that *Daphnia* prioritize resource allocation to maintenance, growth and reproduction, thus under limited food conditions reproduction will be affected first, then growth and ultimately maintenance and hence survival (Glazier, 1992). The high food ration levels used in the present study and those recommended by the OECD are not limiting (1–1.5µg C/mL, (Barata and Baird, 1998), but those remaining in the water column after nanoparticle sedimentation including that used for the low food ration level (0.3µg C/mL) became limiting. Accordingly, except for the micron-sized Tiona particle suspensions of 1mg/L at high food ration levels, the studied particle suspensions impaired reproduction and fitness (measured as r) in *D. magna* in the semi-static test performed with larger bottles (Figure 5.7 left panel graphs). At limiting low food ration levels (i.e. low food experiment) the studied particle suspension by decreasing food concentrations remaining in the water column dramatically impaired *D. magna* reproduction and fitness even at 1mg/L of particle suspensions. Indeed, observed life-history effects were similar than measured in individuals cultured across food ration levels matching those not sedimented and remaining in the water column (i.e. food experiment). At high food ration levels, life-history effects of nano-TiO₂ were only evident for P-25 at 10mg/L, thus, contradicting food depletion effects reported in Figure 5.4 that predicted limiting food conditions and hence detrimental effects on life-history responses at 1 and 10mg/L for all nano-TiO₂. Therefore at low food ration levels nanoparticles acted mainly by reducing food availability. The lack of match observed at high food ration levels between expected and observed effects due to food depletion are likely related to the fact that *D. magna* individuals are able to move across the water column (Ringelberg, 1999; Young and Getty, 1987) and hence could feed on sedimented nanoparticle-algae aggregates from the bottom of the test vessels. The higher proportion of nanoparticles versus algae or/and the use of very limiting food conditions (Barata and Baird, 1998) may explain why at low food ration *D. magna*

individuals were truly starving even if grazing on sedimented algae–particle aggregates.

Life-history effects observed in the re-suspension experiment allowed us to indirectly test effects of the studied nano-TiO₂ in food ingestion and assimilation rates since tests were conducted under continuous re-suspension of algae and nanoparticles, thus minimizing the effect of food and nanoparticle depletion from the water column. Results depicted in Figure 5.7 in the re-suspension experiment and under high food ration levels (right panel graphs) showed that only the nano-TiO₂ P-25 at 10mg/L impaired growth, reproduction and fitness (*r*) similarly to that in the semi-static test (Figure 5.7 left panel graphs). These results indicate that at high food ration levels the P-25 may affect the feeding process decreasing food intake and hence growth and reproduction. In the re-suspension experiment performed at low food ration levels, nano-TiO₂ affected reproduction and fitness (*r*) only at high exposure levels of 10mg/L. There is reported evidence that P-25 nanoparticles at high doses (50mg/L) clog the gut of *C. dubia* and diminished its energy intake (Li *et al.*, 2011). Our data, thus, indicate that the studied particle suspension at high exposure levels of 10mg/L and under limiting food conditions affected food acquisition and hence fitness probably by clogging the digestive tract. The exception was the nano-TiO₂ P-25 that affected fitness responses negatively at low and high food ration levels. Interestingly the nano-TiO₂ P-25 was the only studied nanoparticle having a phase composition dominated by anatase, which is phototoxic under UV light (Sayes *et al.*, 2006). Thus, despite of using artificial light with low UV emission (<10W/cm² of UVA), photocatalytic oxidative stress effects of nano-TiO₂ P-25 cannot be ruled out (Colvin, 2003; Klaine *et al.*, 2008). Note, however, that the UV-A intensity in the present study was about 170-fold lower than in those that reported nano-TiO₂ photo-toxicity in *D. magna* (Ma *et al.*, 2012).

Results obtained at low food levels in semi-static experiments together with the high correlation values observed between fitness (*r*) responses and the size of particle–algae aggregates or sedimentation rates (see Table 5.3) indicate that measured effects on growth, reproduction and fitness are likely to be related to depletion of food. Despite that the studied nano-TiO₂ can also affect growth and reproduction by clogging the gut, this phenomenon was measured under re-suspension conditions and at high exposure levels of 10mg/L. At high

food ration levels, however, our experimental design failed to predict life-history effects of the studied nano-TiO₂. The more plausible explanation was that our nanoparticle food depletion hypothesis was tested assuming that *D. magna* individuals do not move in the water column and hence cannot graze from the bottom of the experimental bottles, which it is not the case for most zooplanktonic organisms (Ringelberg, 1999).

There is still substantial controversy on the mode of toxic action of nanoparticles to aquatic organisms due to problems, for example, to establish exposure routes (Handy *et al.*, 2012b). Most nanotoxicity studies follow conventional protocols designed to test dissolved compounds instead of toxic particle suspensions (Handy *et al.*, 2012b). For example in the semi-static testing conditions of OECD protocols (OECD, 1981), nanoparticles are likely to aggregate with themselves and with algae, increasing algae sedimentation rates and hence diminishing food levels for grazers and ecotoxicological model organisms such as *D. magna*, *C. dubia* or the planktonic copepod *Acartia sp.* that graze algae from the water column (Barata and Baird, 2000; Barata *et al.*, 2012; Barata *et al.*, 2002). In relation to this, test vessel volumes usually used to conduct chronic tests with planktonic grazers are quite small, hold small water columns, hence minimizing algae sedimentation effects. The above mentioned limitations, however, can be improved using larger enclosures holding larger water columns under semi-static conditions. Here we present data clearly showing that at limiting food conditions the use of larger enclosures allowed to assess food depletion effects on the studied zooplanktonic species *D. magna* that were not detected in the standardized reproduction *D. magna* tests of the OECD (Figures. 5.6 and 5.7). Indeed, in the OECD reproduction test depicted in Figure 5.6 effects of the studied nano-TiO₂ on fitness (*r*) were only observed at low food ration levels for NM-103 at 10mg/L, whereas in our semi-static test effects on *r* were observed in 7 out of the 12 treatments of nano-TiO₂ and algae (Figure 5.7). These results agree with those of (Seitz *et al.*, 2012) that using similar food conditions and small volumes (50 ml) found no effects of P-25 on *D. magna* reproduction at ≤ 2 mg/L. Therefore our data clearly support the need to implement new test design for assaying nanoparticle toxicity in aquatic biota.

In summary despite that many studies have already addressed the toxicity of nano-TiO₂ in *D. magna* and other planktonic species, few of them have reported

clear evidences that nano-TiO₂ aggregate to algae, diminish food levels and hence dramatically affect reproduction and fitness of zooplanktonic species.(Li *et al.*, 2011) studying the effects of two types of metallic nanoparticles (TiO₂ and Al₂O₃) in growth and reproduction of the cladoceran *C. dubia* showed that these two nanoparticles aggregated to algae, depleted food levels and impaired growth and reproduction of the studied cladoceran species but at concentrations greater than 10mg/L. In this present study we showed that nanoparticle concentration and food level are important parameters to be considered and that there was a clear relationship between particle–algae aggregate size, food depletion rates and effects on fitness. This present study, however, was conducted under relatively high concentrations of nano-TiO₂ ($\geq 1\text{mg/L}$), which are greater than those predicted to be found in the environment (Gottschalk *et al.*, 2009). Nevertheless, particle–cell aggregation is expected to increase with exposure time in waters with high ionic strength, thus in field conditions even low concentration levels of nano-TiO₂ may aggregate to algae making them to settle, a process that occur in other nanoparticle types (Hoecke *et al.*, 2009; Li *et al.*, 2011). Depletion of food levels from the water column may have dramatic consequences to the planktonic aquatic food web (Baird and Burton, 2001). Thus, future efforts should be focused in studying such effects using realistic nanoparticle suspensions, longer periods and probably larger experimental enclosures.

5.5. Acknowledgements

The authors acknowledge the help and support of the National Centre for Electron Microscopy, at the Complutense University of Madrid and to José Manuel Fortuno~ at the Marine Science Institute from Barcelona in all the electron microscopy work for determining the particle sizes and algae–particle aggregate images. This project was funded by the Spanish projects CTM2011-30471-C02-01 and AEG 07-060.

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CHAPTER 6

**First evidence for toxic defence in *Daphnia magna* by
the multixenobiotic resistance mechanism**

First evidence for toxic defence in *Daphnia magna* by the multixenobiotic resistance mechanism

Bruno Campos¹, Rolf Altenburger², Cristian Gómez¹, Silvia Lacorte¹, Benjamin Piña¹, Carlos Barata¹, Till Luckenbach²

¹Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona 18, 08034 Barcelona, Spain

²UFZ-Helmholtz Center for Environmental Research, Permoserstr.15, D-04318 Leipzig, Germany.

Submitted Aquatic Toxicology

Abstract

Chemosensitization, which is reduction of tolerance of organisms against toxicants by inactivation of cellular multixenobiotic resistance mechanisms (MXR), could provide a mechanistic explanation of cases where combined effects of a mixture of compounds are stronger than the added effects of the isolated constituents. We cloned partial cDNA sequences of four MXR related ABC transporter genes in the water flea *Daphnia magna* and found constitutive expression of the respective transcripts in *D. magna* eggs, embryos, neonates and juveniles. Increased levels of transcripts of two out of the four newly sequences genes were detected in juveniles exposed to known inducers (mercury and pentachlorophenol). MXR associated efflux activity was monitored using the fluorescent substrate dyes rhodamine 123, rhodamine B and Calcein-AM combined with specific MXR transporter inhibitors (chemosensitizers). Specific inhibitors of ABCB1 and/or ABCC transport activities (reversin 205, MK571 and cyclosporine A) resulted in a concentration dependent inhibition of the transport of fluorescence dye reporters in *D. magna* juveniles. Furthermore, all the studied inducers (mercury, pentachlorophenol, dacthal) decreased dye accumulation in pre-exposed juveniles, which were interpreted as induction of transporter activity. Mitoxantrone and chlorambucil, putatively toxic ABCB1 and ABCC substrates, respectively, were applied singly and in combination with the specific chemosensitizers to study the tolerance role of ABCB and ABCC efflux transporters. In five out of the eight binary combinations between the studied chemosensitizers and toxic substrates joint toxicities were greater than additivity. These results evidenced the existence of the genes and of the associated efflux activities of ABC transporters in juvenile stages of *D. magna* and indicate that MXR mechanisms may play a significant role in the species' tolerance to environmental contaminants.

Keywords: *Daphnia*, mixture toxicity, phospho-glycoprotein, multidrug resistant protein.

6.1. Introduction

The cellular multixenobiotic resistance (MXR) system in aquatic organisms represents a broad-scale defence mechanism protecting cells and organisms against environmental toxicants dissolved in the water (Kurelec, 1992). MXR is mediated by membrane-based transport proteins from the ATP binding cassette (ABC) protein family, which recognize a wide variety of chemical structures as substrates, pumping them out of the cell in an energy dependent, ATP-driven process thus keeping their levels in cells low. Cellular MXR efflux activities in various aquatic invertebrate species were so far associated with homologs of mammalian ABCB1 and ABCC proteins (Bard, 2000; Epel *et al.*, 2008a).

There are two aspects regarding MXR of aquatic organisms that are highly important for assessments of ecotoxicological effects of chemicals:

1) Organisms with potent MXR defence are less sensitive to the toxic impact of compounds that act as transporter substrates, because comparatively few of those molecules reach their site of toxic action (Bard, 2000; Epel *et al.*, 2008b). In comparison, non-substrates will appear more bioactive because relatively more of those molecules are bioavailable. Consequently, bioassay-based assessments of the ecotoxic potentials of chemicals will therefore be biased: compounds that are substrates of MXR transporters of the test species used will appear less hazardous than non-substrates, even though they may have similar bioactive potentials when internal concentrations and number of molecules reaching their target site are corresponding.

2) Compounds can act as inhibitors of MXR transporters, causing increased sensitivity of organisms towards other, toxic compounds that, as substrates, are usually *kept out of the organism*. This so called chemosensitizing effect is a possible mechanism behind synergistic effects of compound mixtures (Kurelec, 1997).

The water flea *Daphnia magna* is a widely used eco-toxicological model species for an aquatic invertebrate (Barata *et al.*, 2002a), but there is so far no evidence in the literature for activity of MXR transporters in daphnids. However, for reasons outlined above information to which degree MXR can affect the bioactive potential of compounds would be important to take into account.

Sixty four ABC transporters were annotated based on genomic sequences of another *Daphnia* species, *D. pulex*, the only crustacean genome so far sequenced (Colbourne *et al.*, 2011). ABC transporter orthologs from all so far characterized subfamilies, ABCA – ABCBH, were identified in the *D. pulex* genome. From the ABCB and the ABCC subfamilies, sequences of homologs of MXR related ABC transporters ABCB1 or P-glycoprotein with two homologs in *D. pulex* and of ABCC1 to 5 or MRP1 to 5 (multidrug resistance-associated proteins) were identified (Sturm *et al.*, 2009).

There are no published studies yet providing experimental evidence that functional properties of these *Daphnia* ABCB and ABCC transporters are indeed MXR related. However, considering that MXR related functions are conserved properties of ABCB1 and ABCC homologs across aquatic animal species (Bard, 2000; Epel *et al.*, 2008a) a similar function may also be assumed for the *Daphnia* homologs (Sturm *et al.*, 2009). ABCB1- and ABCC-like efflux activities in daphnids should therefore become visible with substrate/inhibitor compounds generally used as probes for MXR transporter activity (Epel *et al.*, 2008a).

This study aimed to determine the role of ABC transporter based efflux activity in *D. magna* as toxic defence against chemicals in the water applying the following approaches:

- 1) Sequences of putative MXR transporters were identified in the *D. pulex* genome, but it has so far not been shown that those genes are indeed active, i.e., transcribed. Using polymerase chain reaction (PCR) techniques constitutive expression of transcript levels of those genes from the ABCB and ABCC subfamilies were determined in different life stages of *D. magna* and changes in transcript levels were quantified in animals upon exposure to known chemical inducers of ABC transporter expression.
- 2) For determining whether MXR transporters are active in *D. magna*, dye efflux assays were performed using fluorescent substrate dyes in combination with specific inhibitor compounds known to interact with MXR transporters in other organisms. Active efflux of those compounds, and thus of MXR transporter activity, is indicated by increased accumulation of dye in the organism with inhibitors present. Dye efflux activity can thus be used as a measure of transporter protein levels: With more protein present, less dye will

accumulate in the cells. Hence, in addition to untreated animals with constitutive transporter protein expression levels, dye efflux assays were also performed with animals previously exposed to known inducers of ABC transporters to investigate changes of protein expression.

3) The activity of MXR transporters was further determined by performing toxicity assays with toxic transporter substrates and inhibitors. The experiments were based on the assumption that, if MXR transporters are active in daphnids, the combination of substrates and inhibitors would cause larger toxic effects than would be anticipated from the effects of compounds when applied singly. Toxic effects were analysed using the independent action (IA) and concentration addition (CA) concepts for dissimilar and similar acting compounds, respectively (Altenburger *et al.*, 2003).

6.2. Material and Methods

6.2.1. Chemicals

Cyclosporine A (CsA), Reversin 205 (REV205), MK571, mitoxantrone, chlorambucil, rhodamine B (RhB), rhodamine 123 (Rh123), calcein AM (Ca-AM), Calcein disodium salt, mercury chloride ($HgCl_2$), dacthal (1, 5 μM) and pentachlorophenol (PCP) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetone, DMSO, (analytical grade), methanol, acetonitrile (SupraSolv grade) and HPLC water (LiChrosolv grade) were supplied by Merck (Darmstadt, Germany). Ammonium formate $\geq 99\%$ were purchased from Sigma-Aldrich (St. Louis, USA) and formic acid were supplied by Merck (Darmstadt, Germany).

6.2.2. Experimental animals

Daphnia magna, clone B obtained from Bayer CropScience, Mannheim, Germany, was used for all experiments. Individual cultures of 10 animals/L were maintained in ASTM hard synthetic water (ASTM, 1980) with the addition of a standard organic extract (Baird *et al.*, 1989). Individual cultures were fed daily with *Chorella vulgaris* Beijerinck (5×10^5 cells/mL). The culture medium was changed every other day, and neonates were removed within 24 h. Photoperiod was set to 14h light: 10h dark cycle and temperature at $20 \pm 1^\circ C$.

6.2.3. Identification of partial *abcb*¹ and *abcc* coding sequences from *D. magna*

In order to obtain partial coding sequences (cds) of *D. magna* ABC transporters, reverse transcription polymerase chain reaction (RT-PCR) was performed with primers designed based on regions encoding the highly conserved nucleotide binding domains (NBD) in *abcb* and *abcc* genes of *D. pulex* (Sturm *et al.*, 2009). Primers (Table 6.1) were designed based on genomic sequences encoding the predicted proteins Dappu-347264 (NCBI accession number EFX85237; ABCB1 homologue), Dappu-347281 (NCBI acc. no. EFX72783; ABCC1-3 (MRP1-3) homologue), Dappu-347288 (NCBI acc. no. EFX68457; ABCC4 (MRP4) homologue), and Dappu-347292 (NCBI acc. no. EFX72656; ABCC5 (MRP5) homologue).

PCR was performed using a MiniCyclerTM (MJ Research) with cDNA from total RNA isolated from *D. magna* juveniles as described below. PCR conditions were 95°C for 5min, followed by 35 cycles at 95°C for 30sec, 55°C for 60sec and 72°C for 30sec each, and a final step at 72°C for 10min. The PCR product was cloned into vector pTZ57R/T from the InsTAcloneTM PCR Cloning Kit (Fermentas) and propagated using X Blue competent cells. Sequencing of cloned DNA was performed on a 3730 DNA Analyzer (Applied Biosystems). Homology of the amino acid sequences predicted from obtained partial *abcb1*, *abcc1-3*, *abcc4*, *abcc5* transporter cds with ABC transporter sequences from other organisms was confirmed using NCBI (National Center for Biotechnology Information, Bethesda, MD, US) by blastn and blastx and the sequence was deposited on GenBank (accession no.KC122929, KC122922- KC122924 for *D. magna abcb1*, *abcc-3*, *abcc4*, *abcc5*, respectively). Alignments were performed with respective sequences of a range of Abcb/ABCB and Abcc/ABCC amino acid sequences from various organisms using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and percent identities were determined. Homologies of the obtained ABC gene sequences were further analyzed using phylogenetic trees with the first 100 hits of BLASTP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) removing predicted and unidentified

¹ Our nomenclature for gene/protein names is: *abcc*/Abcc for *Daphnia* and ABCC/ABCC for human

sequences. Phylogenetic trees were made using JSTREE (<http://lh3lh3.users.sourceforge.net/jstree.shtml>).

Table 6.1. Primer pairs designed from *D. pulex* used for amplification of *Daphnia magna* ABCB and ABCC subfamilies partial sequences. Primers used to amplify reference *D. magna* genes are also included.

Genes	Forward	Reverse	Amplicon
PCR primers			
ABCB	CGTCGCCTGGTCGGCACTTC	CAGCCTGTGGCGACAATGATGGTG	502
ABCC1-3	CAGAGGGTGGCGAGAATTAA	GGCGCGTATTCTTGATTTC	259
ABCC4	AGGAACCTGTCGAATTGCTG	ATTGAAGCTGGTGGGTGACT	453
ABCC5	ACGTCAAAATGCGCTACAGA	TCAGTACGGACGGTTGTCA	165
Real time qPCR primers			
ABCB	GTATCCAGTGCAGGAAGTGGC	ACAGCGTATCGCTATTGCC	100
ABCC1-3	TAGCTCGCGCTACTGAGAA	GATCGTCGGTCTCCAGATCG	100
ABCC4	CCCGATCCCTTACGTCGAT	GGTGGCGTCCTACATGAGTGT	100
ABCC5	CAGTCCAGTCATCGAGAACGG	TGACGCAACAGAGCTCGG	100
GAPDH	GACCATTACGCTGCTGAATACG	CCTTGCTGACGCCGATAGG	100
β-actin	TTATGAAGGTTACGCCCTGCC	GCTGTAACCGCGTTCAGTCAA	100
18S	CGCGAATGGCTCAATAATCA	CAGTCGAGGCTCGAGTGCA	100

6.2.4. RNA extraction and qPCR analysis

Abundances of *D. magna* *abcb*, *abcc1-3*, *abcc4* and *abcc5* transcripts were determined in just released eggs (stage 1 eggs), embryos (stage VI) (Threlkeld, 1979), newly released neonates (<12h) and 4 day old juveniles. Eggs and embryos were obtained from de-brooding females by gently flushing water to the brood chamber (Glazier, 1992). For RNA extraction 20 eggs, 20 embryos, 10 neonates and 5 juveniles were pooled to obtain sufficient RNA. Total RNA was isolated from *D. magna* tissues using Trizol reagent® (Invitrogen™). The RNA concentration was measured by spectrophotometric absorption in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, DE) and the quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). Quantities from 1 µg to 100 ng of DNase II-treated RNA (Ambion®) were retro-transcribed to cDNA using the First Strand cDNA Synthesis Kit (Roche Applied Science®) and stored at -20°C. The amounts of cDNA used for quantitative real-time PCR (qPCR) corresponded to 10 ng of the original RNA preparation. qPCR was performed with a LightCycler® 480 Real-Time PCR System using LightCycler® 480 SYBR Green I Master

(Roche Applied Science®). qPCR primers (Table 6.1) were designed with Primer Express software. Sequences of amplicons were confirmed by sequencing using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems) and by comparing them to the corresponding references in GenBank using NCBI BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

Relative mRNA abundance values were calculated from the second derivative maximum of their respective amplification curve (C_p , values from triplicate assays). RNA abundances of putative reference genes β -*actin* and *g3pdh* (mRNA) and of 18S (rRNA) (Heckmann *et al.*, 2006) were quantified and tested for stability in the three RNA sources used in this work, eggs, embryos and juveniles. Table 2 shows average C_p values and their standard deviations from all samples tested in this work (each sample tested in triplicate). The minimal variation of C_p values was found for 18S rRNA, which showed SD values below 1 C_p units in all samples; therefore, this gene was selected as reference gene.

C_p values obtained for the target genes (TG) were compared to the corresponding values of 18S to obtain ΔC_p values ($\Delta C_p = C_p \text{ ref} - C_p \text{ TG}$). PCR efficiency values for reference and target genes were calculated as described by Pfaffl (2001a) and assumed to be close to 100% from these calculations. qPCRs were performed with RNA extracts from nine replicates with three runs per sample. Mean mRNA abundances and standard deviations were calculated for each gene from the mean values of each sample.

6.3.5. Dye efflux assays

MXR transporter activities were determined in 4-day old juveniles using dye accumulation assays. The assays are based on the property of certain fluorescent dyes to act as substrates of ABC efflux transporters. The dyes are kept out of cells if efflux transporters are active and increasingly accumulate inside the cells if transporter activities are disrupted by transporter inhibiting chemicals (Homolya *et al.*, 1993; Neyfakh, 1988). As proxies for efflux activity in daphnids we used two rhodamine dyes, RhB and Rh123, and Ca-AM. Ca-AM is not fluorescent but forms fluorescent Calcein once it reaches the cytosol where it is cleaved by cellular esterases. All dyes are known to be transported by mammalian ABCB1 and ABCC transporters (Daoud *et al.*, 2000; Essodaïgui *et*

al., 1998; Holló et al., 1994; Homolya et al., 1993; Neyfakh, 1988; Yeheskely-Hayon et al., 2009a). Low fluorescence levels of cells/tissues indicate high efflux transporter activity and enhanced fluorescence occurs with low efflux activity. For disrupting ABCB1 and ABCC type efflux activities in tissues from different live stages of *D. magna* we used the model inhibitors of mammalian ABCB1 and ABCC transporters, REV205 (Sharom et al., 1999a) and MK571 (Gekeler et al., 1995), respectively, and the inhibitor of both transporters CsA (Qadir et al., 2005).

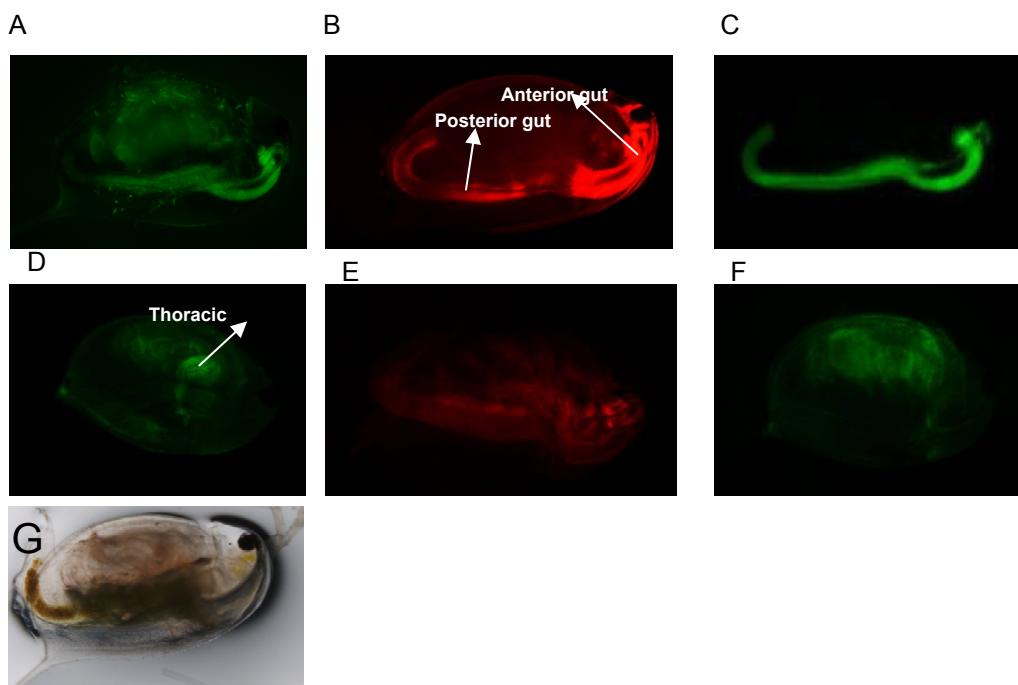


Figure 6.1. Fluorescent microscopy images of *D. magna* juveniles exposed to Rh123 (A,D), RhB (B, E), Ca-AM (C,F). Images were taken after 1.5h for *in vivo* (A-C) and 2h for *ex-vivo* (D-F) incubation periods with 5 μ M RhB, 2.5 μ M Rh123 or 0.5 μ M Ca-AM. Images of a juvenile individual taken with the bright file is also included (G).

We noticed that the ABC transporter inhibitors we used had a toxic effect on *D. magna* that, independently from transporter inhibition, changed accumulation of fluorescent dyes in the animals by affecting their filtering activity. Accumulation of fluorescent dyes in vital animals that mainly occurred in the gut of *D. magna* (Fig 6.1) strongly depended on the filtering activity of the animals. Thus, we observed that dye uptake was reduced by 60 % when filtering activity was blocked (Fig 6.2).

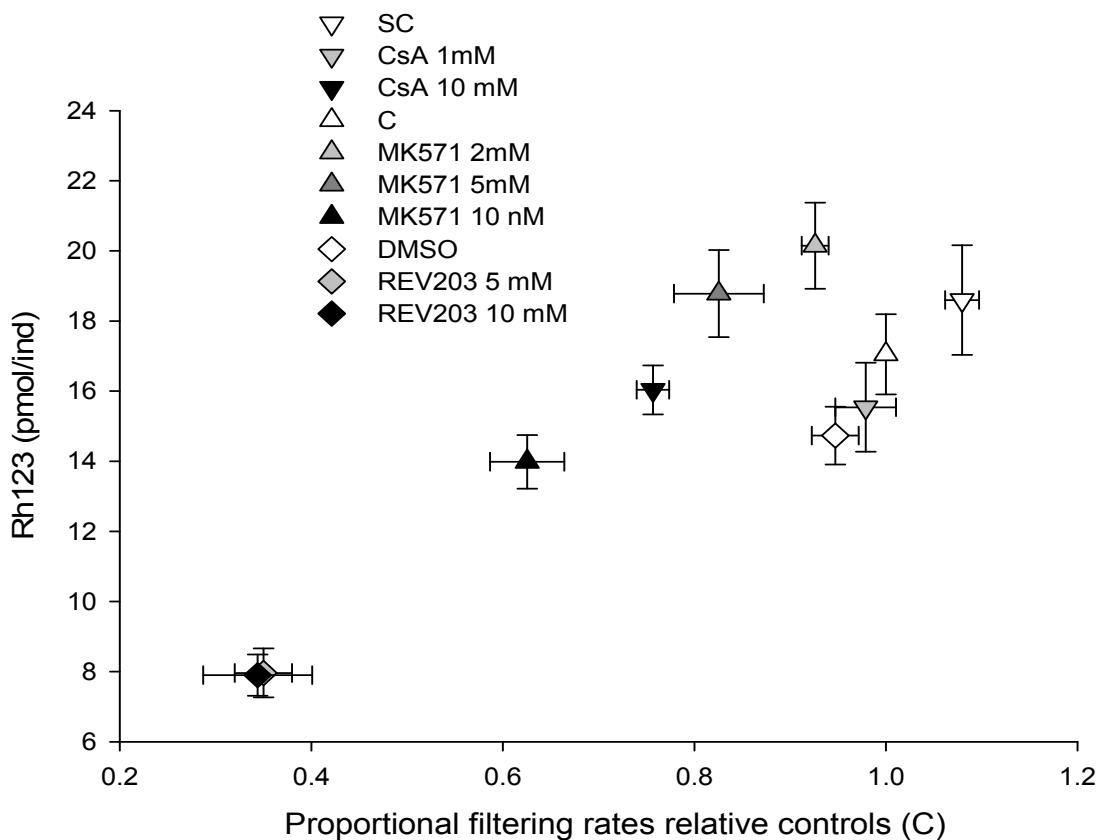


Figure 6.2. Effect of transported MXR inhibitors on the accumulation of Rh123 (pmol/individual) and proportional filtering rates in *D. magna* juveniles. Symbols and error bars are Mean \pm SD ($n=10$).

In order to clearly determine inhibitor effects on ABC transporter related efflux activity in *D. magna*, animals were immobilized immediately prior to dye efflux assays by pinching their hearts with a needle and thus stopping filtering activities in all animals used in the experiment. Cells and tissues in these animals were still vital and dye efflux activity was intact. This “ex-vivo” experimental procedure where vital tissue instead of intact animals is used for recording MXR transporter activity can be seen as similar as other approaches where freshly dissected bivalve gills were used instead of whole organisms (Cornwall *et al.*, 1995; Faria *et al.*, 2011a; Luckenbach and Epel, 2005b).

Immobilized *D. magna* juveniles (4 days old) were exposed individually for 1h to 2.5, 1.5 and 0.5 μ M of Rh123, RhB and Ca-AM (stock dissolved in DMSO), respectively, in 110mL of ASTM hard water. In parallel to controls with dye only and solvent controls with dye and solvent, treatments with 1-10 μ M of model inhibitors CsA, MK571 and REV205, and the transporter substrates chlorambucil (20, 200 μ M) and mitoxantrone (2, 10 μ M) were run. All controls

and treatments were replicated 10 times. After the exposure period animals were transferred to 100mL clean ASTM hard water for 5min to allow depuration of excess dye, and the accumulated dye was extracted from tissue by sonication of animals in 1mL of ASTM water. Fluorescence measurements were performed in a microplate fluorescence reader (Synergy 2, BioTek, USA) using excitation/emission wavelengths of 480/530nm for Rh123 and Ca-AM and 530/590nm for RhB. Measurements of each replicate were run in triplicates and were corrected for background fluorescence levels of ASTM water. Fluorescence values were then converted to concentration units using a calibration curve based on 8 data points from different concentrations of calcein disodium salt (fluorescent dye), or Rh123 or RhB. Accumulated dye is reported as pmol/individual.

6.3.6. Gene and dye efflux induction experiments

D. magna juveniles (4d old) were pre-exposed to two sublethal concentrations of inorganic mercury dosed as HgCl₂ (Hg, 1,10nM) and the pesticides dacthal (1, 5µM) and pentachlorophenol (PCP; 2,8µM). The tested concentrations were selected from preliminary acute responses. These three contaminants at the tested concentration range are known to induce ABC transporter transcripts, proteins and related efflux activities in mussel gills and fish cell lines (Della Torre *et al.*, 2012; Eufemia and Epel, 2000; Navarro *et al.*, 2012b; Tutundjian and Minier, 2007b). Experiments were set up with groups of five individuals in 400mL of ASTM hard water with chemical added in the treatments and without chemical in the controls. Dacthal and pentachlophenol were dosed using acetone (<0.1mL/L) as a carrier and hence appropriate solvent controls were also used. After pre- exposures either transporter efflux activities in the animals were determined with dye efflux assays or transporter transcript levels were quantified with qPCR.

6.3.7 Toxicity assays

Acute toxicity assays were performed with the toxic ABCB1 substrate mitoxantrone (Taylor *et al.*, 1991a), the toxic ABCC1 substrate chlorambucil (Su *et al.*, 1998a), the ABCB1 inhibitor REV205, the ABCC inhibitor MK571, the ABCB1/ABCC inhibitor CsA and with combinations of toxic transporter

substrates and inhibitors. The toxicity assays with *D. magna* were performed following established guidelines (OECD, 1981). For preparing stock solutions, CsA and chlorambucil were dissolved in acetone, REV205 was dissolved in DMSO and MK571 and mitoxantrone were dissolved in milliQ water. Appropriate amounts on the chemical stocks were then added to ASTM hard water to prepare test solutions. The same amount of carrier (acetone or DMSO, 0.1mL/L) were added to a solvent control and to the rest of test solutions to account for any carrier effect on survival and an additional control (ASTM water only) treatment were used. No mortality, monitored as immobile individuals, was observed in either carrier controls or non-manipulated controls.

In a first set of experiments, toxicities of single compounds were determined with 6 to 8 concentrations in duplicates. Based on the concentration-effect curves derived from these data concentrations applied in binary mixtures of toxic substrates and inhibitors were defined and predictions of the combined toxic effects were made. In a second set of experiments, toxicities of binary mixtures of the toxic substrate compounds mitoxantrone and chlorambucil were determined, each combined either with inhibitor REV205, MK571 or CsA. We hypothesized that pairings of substrate and inhibitor compounds that interact with the same efflux transporter type (ABCB1-type efflux: mitoxantrone with REV205 or CsA; ABCC-type efflux: chlorambucil with MK571 or CsA) had joint toxic effects that were greater than additive because interference of inhibitors of a specific transporter type will result in increased uptake and toxic effect of the respective toxic substrate. Further, as the compounds that we used as inhibitors are in some cases also transporter substrates as was shown for CsA, a substrate of ABCB1 (Saeki *et al.*, 1993), greater than additive toxic effects may also be expected for pairings of inhibitors targeting the same transporter type (ABCB1-type efflux: REV205 with CsA; ABCC-type efflux: MK571 with CsA) but not for the pairing of the inhibitors REV205 and MK571 targeting different transporter types. Additive joint toxicity was expected for pairings of both toxic substrate compounds (mitoxantrone, chlorambucil) and of the ABC transporter inhibitors REV205 and MK571. Toxicities of the mixtures were determined using the ray design that is based on constant mixture ratios deduced e.g. from LC50 values (Altenburger *et al.*, 2003). The ray design enables to determine whether

toxicities of mixtures follow the “concentration addition (CA)” or “independent action (IA)” concepts.

6.3.8. Determination of the stability of test chemicals in aqueous test solutions

The stability of mitoxantrone, chlorambucil, CsA, MK751, and REV205 in ASTM hard water over the duration of the toxicity tests was investigated by measuring their concentrations in freshly made aqueous test solutions (time 0) and after 24 and 48 h . Two concentrations of each compound were tested that corresponded to low and high exposure levels in the toxicity tests (0.5-10mg/L) (Table 6.2). Experiments were conducted under the same conditions as toxicity assays. Dissolved levels of the studied compounds were determined by direct sample injection using an AcQuity Ultra Performance Liquid Chromatography (UPLC) system equipped with a quaternary pump and connected to a triple quadrupole MS/MS system (Waters, USA). Data was acquired and processed using MassLynx 4.1 software package. A ZORBAX Eclipse XDB-C18 Narrow-Bore column 2.1 x 150mm, 5µm from Agilent Technologies (Santa Clara, USA) was used. The mobile phase consisted of solvent A 5mM ammonium formate in water and solvent B 5mM ammonium formate in methanol. The initial mobile phase composition was 70% A and 30% B (5min), to 70% B in 3min and held for 10min, and to 100% B in 3min. These conditions were held for 4min and then the initial conditions were regained in 5min with an equilibration time of 2min. The flow rate was set at $0.3\text{mL}^*\text{min}^{-1}$. To optimize ionization and to establish mass spectral features, individual standards were first analysed by Flow Injection Analysis (FIA) in UPLC-MS in positive ESI mode, except for CsA that was analysed in negative ESI mode. Full-scan data acquisition was performed scanning from m/z 100 to 600 in profile mode, using a scan time of 2s with a step size of 0.1u and a pause between each scan of 2ms. Optimized parameters were source temperature (from 125 to 150°C), cone voltage (from 5 to 50V) and collision energy (from 5 to 50eV).

6.3.9. Data analyses and modelling responses

One way ANOVA analyses of relative mRNA expression was performed using ΔCp values, as this parameter followed normal distribution and the

variances were homocedastic, as assessed by the Kolmogorov–Smirnov, Shapiro Wilk and Barlett's tests. To facilitate the interpretation of results these values were expressed as mRNA copies of target gene per 10^6 copies of the reference gene mRNA.

Transporter efflux activity was evaluated comparing the levels of fluorescence in absence and presence of inhibitors across treatments using one way ANOVA. Lowest Effect Concentration values (LOEC) were estimated with post-hoc Dunnet's test. When required data was log transformed to improve ANOVA assumptions of normality and variance homocedasticity. Statistics tests were performed using the SPSS 17 (SPSS Inc., 2002) package.

6.3.9.1. Modelling single and mixture effects

Transporter efflux activity and IC₅₀ values were evaluated by fitting data to the classical Hill four parameter dose–response model (eq. 6.1)

$$R = \frac{\min + (\max - \min)}{1 + \left(\frac{IC_{50}}{x} \right)^Hill} \quad (\text{eq. } 6.1)$$

where R is the response, min and max represents minimum and maximum of the response, $Hill$ is the shape parameter, IC₅₀ is the concentration of chemosensitizers that corresponds to 50% of the maximal effect.

The concentration–response relationships of the individual substances were biometrically modelled by using a best-fit approach (Scholze *et al.*, 2001) and eq 6. 2 which is a simplification of eq. 6.1:

$$E(\%) = \frac{100}{1 + (LC_{50}/x)^p} \quad (\text{eq. } 6.2)$$

with E = effect in %; p = slope; LC = lethal effect concentration; x =concentration (μM).

On the basis of the concentration–response functions of individual compounds, predictions of concentration addition were calculated for mixtures containing binary combinations in a definite ratio (based on LC₅₀). A total concentration of the mixture, at which a certain effect is generated, can be calculated using concentration addition according to eq. 6.3

$$LCx_{\text{mix}} = \left(\sum_{i=1}^n \frac{p_i}{LCx_i} \right)^{-1} \quad (\text{eq. } 6.3)$$

In this equation LCx_{mix} is the total concentration of the mixture provoking $x\%$ lethal effect; $LCxi$ is the concentration of component i provoking the $x\%$ lethal effect, when applied singly; and p_i denotes the fraction of component i in the mixture. The calculation of total mixture concentrations for various effect levels lead to a complete iteration of an expected concentration–effect relationship.

The prediction concept independent action allows explicit calculation of combined effects according to eq. 6. 4

$$L(c_{\text{mix}}) = 1 - \prod_{i=1}^n (1 - L(c_i)) \quad (\text{eq. } 6.4)$$

The lethal effect at the total concentration of the mixture, $L(c_{\text{mix}})$, is based on the lethal effects of the components which they generate at concentration x at which they are present in the mixture ($L(c_i)$). If the latter is expressed as a fraction (p_i) of the total mixture concentration, it holds eq. 6.5:

$$L(c_{\text{mix}}) = 1 - \prod_{i=1}^n (1 - L(p_i c_{\text{mix}})) \quad (\text{eq. } 6.5)$$

This allows calculation of an effect expected according to the concept of independent action for any concentration of the mixture.

6.4. Results and Discussion

6.4.1. Stability of test chemicals in aqueous test solutions

Concentrations of REV205, MK571 and CsA in unmodified form were stable in ASTM hard water over 48h as measured concentrations at 0, 24 and 48h were close to nominal ones (Table 6.2). REV205 was confirmed with a fragment ion at m/z 699, formed by the loss of ester moiety. MK571 was confirmed with the fragment ions at m/z 409 and m/z 382, and CsA was confirmed with fragment ions at m/z 513 [C₂₅H₄₇N₆O₅]⁺ and m/z 113 [C₇H₁₃O]⁺ (Table 6.2). Mitoxantrone was rapidly degraded, the parental compound was absent even in freshly prepared aqueous solutions. The two identified transformation products were TP1 at m/z 268 and TP2 at m/z 282, both confirmed with the fragment ion at m/z 214. TP1 at m/z 268 corresponds to [C₄H₁₀NO]⁺. TP2 identified at m/z 282 corresponds to the loss of N-ethylethanolamina [C₄H₁₀NO]⁺ and N-methylethanolamina [C₃H₈NO]⁺. These two TPs that were stable during the 48h period of the experiment in sum corresponded to almost 100% of initial

nominal concentrations (Table 6. 2). Chlorambucil quickly degraded in aqueous solutions. The parental compound, monitored at m/z 304, was detected only in the freshly prepared solutions, albeit at a level 81 % below the nominal concentration in the high concentration solution. The transformation product of chlorambucil [C₁₄H₁₈NOCl₂]⁺ was identified at m/z 286, corresponding to the loss of the hydroxyl group.. It accounted for 95% and 85 %, respectively, of the nominal concentrations in the freshly prepared solutions, but was absent after 24 and 48 h (Table 6.2).Our results agree with other studies showing that the mitoxantrone and chlorambucil parental compounds rapidly degraded to transformation products in aqueous solutions (Löf *et al.*, 1997; Lohmann and Karst, 2007; Negreira *et al.*, 2013). There are no reports on toxicities of transformation products of those cytostatic drugs (Kosjek and Heath, 2011). Nevertheless, further toxicological studies conducted in our lab (data not shown) were performed using water initially fortified with toxic doses of mitoxantrone and chlorambucil and subsequently left aging during 4 days (Gómez-Canela *et al.*, in press). The results of those experiments showed that D. magna toxicity of aqueous solutions of mitoxantrone were stable for 96 hours, whereas those of chlorambucil decreased exponentially during the first 24h. This means that mitoxantrone toxicity or that of its transformation products was stable over the studied period but that of chlorambucil did not decreasing to marginal levels within the first 24h.

Table 6.2. Nominal and measured concentration levels (mg/L) of the studied compounds in ASTM hard water in freshly prepared (time 0) and old test solutions (24–48 h). Protonated molecule of mitoxantrone was not detected and instead its transformation products TP1 and TP2 were analysed. Chlorambucil analyses include protonated molecule and transformation product 1 (TP1). BDL, below detection limit.

Compound	Protonated molecule [M+H] ⁺	Fragment ions	Nominal (mg/L)	Time 0 h		Time 24 h		Time 48 h	
				Mean (mg/L)	SD	Mean (mg/L)	SD	Mean (mg/L)	SD
Mitoxantrone	445	214	2	BDL	--	BDL	--	BDL	--
			10	BDL	--	BDL	--	BDL	--
Mitoxantrone TP1	268	214	2	1.91	0.02	1.90	0.02	1.90	0.02
			10	7.26	0.09	6.98	0.18	7.12	0.15
Mitoxantrone TP2	282	214	2	0.09	0.02	0.10	0.01	0.10	0.01
			10	2.74	0.09	3.02	0.18	2.88	0.15
Chlorambucil	304	181	2	BDL	--	BDL	--	BDL	--
			10	1.52	0.27	BDL	--	BDL	--
Chlorambucil TP1	286	181	2	1.90	0.01	BDL	--	BDL	--
			10	8.48	0.27	0.54	0.08	BDL	--
Reversin 205	799	699	0.5	0.40	0.04	0.41	0.08	0.36	0.08
			5	3.81	0.17	3.44	0.23	4.18	0.42
MK571	515	409/382	1	1.03	0.06	1.19	0.05	1.15	0.05
			5	5.22	0.29	5.13	0.08	5.12	0.21
CsA	1201 [M-H] ⁻	113/513	0.5	0.54	0.15	0.51	0.11	0.42	0.09
			5	4.95	0.32	4.70	0.49	4.00	0.87

6.4.2. Identification and expression of partial ABCB, ABCC cds

RT-PCR with primers directed against abc transporters predicted from the *D. pulex* genome (Sturm *et al.*, 2009) resulted in *D. magna* sequences that were identified as partial *abcb*, *abcc1/3*, *abcc4* and *abcc5* cds (Fig 6.3).

The structure of full transporter ABC proteins, such as ABCB and ABCC, comprises 3/4 subunits, each with one or two trans-membrane domain and two nucleotide binding domains (NBD) (Sturm *et al.*, 2009). The NBDs contain typical and highly conserved motifs, such as Walker A, Walker B and ABC signatures (Ambudkar *et al.*, 2006). For these reason this was the target region of the gene selected for sequencing. We cloned five partial sequences, ranging from 200 to 540bp cDNA sequence from *D. magna* (NCBI access. numbers KC172920, KC172922- KC172924 for ABCB1, ABCC1-3, ABCC4, ABCC5, respectively). Alignments of the obtained *D. magna* partial ABC amino acid sequence with respective NBD sequences from *D. pulex* ABCB, ABCC1-3, ABCC4 and ABCC5 showed a 94%, 99%, 98% and 91% identity, respectively, indicating that the identified sequence encodes partial NBD's of a *D. magna* ABC transporters (Figure 6.3). Obtained sequences encompassed NBD fragments as determined with the Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2011) including ABC signature and Walker B motif (Figure 6.3).

NCBI BLAST of the *D. magna* amino acid sequences further confirmed their identities as different ABC transporter homologs. This is illustrated by the phylogenetic trees based on alignments of the respective *D. magna* amino acid sequences with sequences appearing within the first 100 hits of a NCBI BLAST search (Fig.6.4).

Daphnia magna ABCB1	KC172920	ident	501	VALVGTS CGKSTCVQ LLQRFYDPIEGSVS IDGNEL RDHL LGWL REQM GVVG QEPV LFGT SIGENIC YGRDG VSQE E MERA KEANAHDFI QRQL PRKYD TLVGERGA QLSGGQ KQRI A	148
Daphnia pulex	EFX85237	94%	481 I L T N.N D.M K G	598
Branchiostoma floridae	XP_002608794	74%	314 C.S T .. I TK.M.TL .. HDI.S.NIQ .. QNI .. S .. A.T.A .. S .. A .. T .. I.K .. K .. K .. N ..	431
Crassostrea gigas	EKC23020	75%	283 S .. II .. E .. E.AL .. RNIKN.NTK .. Q.I .. S .. I .. A.T.A .. R .. KEAI .. Q.I.A .. M .. MNF.K .. E .. M ..	400
Rattus norvegicus	AAI07561	76%	421 N .. T .. L .. E .. QDI .. TINVRY .. II .. S .. A.T.A .. R .. EN .. TMD .. I .. K.V .. Y .. MK .. H .. FN ..	538
Rattus norvegicus	AAK83023	75%	415 N .. T .. L .. E .. QDI .. TINVRY .. II .. S .. A.T.A .. R .. EN .. TMD .. I .. K.V .. Y .. MK .. H .. F ..	532
Sus scrofa	AAW02918	76%	155 N .. T .. M .. L .. T .. V .. QDI .. TINVRY .. II .. S .. A.T.A .. R .. EN .. TM .. I .. K.V .. Y .. MK .. N .. F ..	272
Rattus norvegicus	AAS91649	76%	415 N .. T .. L .. E .. QDI .. TINVRY .. II .. S .. A.T.A .. R .. EN .. TMD .. I .. K.V .. Y .. MK .. H .. F ..	532
Rattus norvegicus	NP_596892	76%	415 N .. T .. L .. E .. QDI .. TINVRY .. II .. S .. A.T.A .. R .. EN .. TMD .. I .. K.V .. Y .. MK .. H .. F ..	532
Rattus norvegicus	P43245	76%	422 N .. T .. L .. E .. QDI .. TINVRY .. II .. S .. A.T.A .. R .. EN .. TMD .. I .. K.V .. Y .. MK .. H .. F ..	539
Hetercephalus glaber	EHB12084	76%	372 N .. T .. I .. L .. T .. V .. QDI .. T.NVRY .. II .. S .. A.T.A .. R .. EN .. TM .. I .. K.V .. Y .. MK .. H .. F ..	489
Daphnia magna ABCB	KC172920	identit	147	IARALVRQP KILLDEAT SALDTQSE SVVQ KALDKAR QGRTT IVAHRL 1	
Daphnia pulex	EFX85237	94%	599 A .. 647	
Branchiostoma floridae	XP_002608794	74%	432 N .. E .. AT .. E .. VI .. 480	
Crassostrea gigas	EKC23020	75%	401 KD .. D .. E .. A .. VV .. 449	
Rattus norvegicus	AAI07561	76%	539 N .. E .. A .. A .. E .. VI .. 587	
Rattus norvegicus	AAK83023	75%	533 N .. E .. A .. A .. E .. VI .. 581	
Sus scrofa	AAW02918	76%	273 N .. E .. A .. V .. E .. VI .. 321	
Rattus norvegicus	AAS91649	76%	533 N .. E .. A .. A .. E .. VI .. 581	
Rattus norvegicus	NP_596892	76%	533 N .. E .. A .. A .. E .. VI .. 581	
Rattus norvegicus	P43245	76%	540 N .. E .. A .. A .. E .. VI .. 588	
Hetercephalus glaber	EHB12084	76%	490 N .. E .. A .. V .. E .. VI .. 538	
D. magna ABCC 1-3	KC172922	identit	3	EGGENL S VQRQLICLAR ALLRKTQV LILDEATAAVD LETDDLIQATIRKEFKE GTVITIAHRLNT ILDSNRVMVL DKGEIKEYA	173
[Daphnia pulex	EFX72783	99%	1440 K .. 1496	
Rattus norvegicus	CAA65258	81%	1 V .. KI .. V .. S .. TQ .. EDS .. L .. M .. YT .. I .. R .. 57	
Pongo abelii	XP_002826206	80%	117 V .. KI .. V .. S .. TQ .. EDC .. L .. M .. YT .. I .. Q .. G .. 173	
Culex quinquefasciatus	XP_001862061	86%	333 K .. R .. T .. DC .. L .. M .. DK .. I .. Q .. T .. 389	
Rhipicephalus microplus	AEI91125	76%	55 I .. V .. SRI .. M .. R .. ADC .. I .. V .. YD .. I .. R .. Q .. V .. F .. 111	
Cricetulus griseus	EGV92991	80%	40 V .. KI .. V .. S .. TQ .. EDC .. L .. M .. YT .. I .. VR .. 96	
Macaca mulatta	AFE71101	80%	178 V .. KI .. V .. S .. TQ .. EDC .. L .. M .. YT .. I .. Q .. G .. 234	
Apis mellifera	XP_001123016	82%	176 I .. K .. S .. Q .. DC .. IL .. D .. II .. N .. R .. V .. 232	

Figure 6.3A. Alignment of the putative partial ABCB1, ABCC1-3-like sequences from *D. magna* with ABC nucleotide binding domains (NBD) of several organisms. Amino acid positions of the comparison ABC sequence stretches that align with the *D. magna* ABC sequences are indicated. The conserved motifs ABC signature and Walker B domain are highlighted in light and dark grey respectively. From all the ABC sequences only amino acids diverging from the *D. magna* ABC sequences are given. Protein codes as those of NCBI.

<u>D. magna ABCC4</u>	KC172923	ident	201 ALDKDFSLFPNGDQTAVGGERGVSLGGQKARVNLARSLYVDADIYLMDDPLSAVDTHVGRHLFDKAINGYLRLDKIRVLVTHQLQ	380
Daphnia pulex	EFX68457	98%	521 T	F
Acromyrmex echinatior	EGI58005	75%	569 . KR . . . L.Y.K.I R.I . . . AV.A A . . . K.M . ECV.K . . G.T.I	627
Drosophila virilis	XP_002056519	74%	547 . . ER . . E.L.Y.K.I A IS . . AV.RK L QCMR . . . N.VL	606
Drosophila pseudoobscura	XP_002137937	71%	520 . . ER . . Q.L.Y.K.I A IS . . AV.RR L QCMH . . . SELVI	579
Drosophila persimilis	XP_002019432	71%	520 . . ER . . Q.L.Y.K.I A IS . . AV.RR L QCMH . . . SELVI	579
Drosophila grimshawi	XP_001994164	73%	545 . . ER . . E.L.Y.K.I A IS . . AV.RK L QCMR.F . ED.V	604
Drosophila sechellia	XP_002031608	71%	524 . . ER . . E.L.F.K.I A IS . . AV.RR L QCMR . . . SELVI	583
Tribolium castaneum	EFA04298	73%	546 . . VR . . Q.L.D . . I . . K.A R.I . . . AV.KN L KQ . . C.T.F.A . VI	605
Drosophila mojavensis	XP_002000613	74%	545 . . ER . . E.L.Y.K.I A IS . . AV.RK L QCMR . . . N.VL	604
<u>Daphnia magna ABCC5</u>	KC172924	identit	3 LYTD EAIWEA IERTNM KDKI KALPD KLDS PVI ENGEN FSVG ERQLLC MAR ALLRHS KVRM	182
Daphnia pulex	EFX72656	91%	1275 V Q . . . L.T I	1331
Daphnia pulex	EFX72657	86%	1263 . D.DV E GQ . . . A I	1319
Daphnia pulex	EFX63846	86%	1124 . D.DV G . . . E G . . . A I	1180
Monosiga brevicollis	XP_001748233	69%	241 V.A . . Q . . T.L . . AH.R.A.RR . . E . . A . . V . . N N.RI	298
Ciona intestinalis	XP_002124642	73%	253 . S . . E . . K.L . . H . . PT . . SE . . . ETE.V I II .	311
Nematostella vectensis	XP_001623747	68%	1196 . S . . EL . . K.L . . SHL . . MVSN . . L . . EA . . V I IL .	1254
Saccoglossus kowalevskii	XP_002739851	68%	282 . . AEL.N.L.K.C . . PN . . D . . EQ.E.T . . V M N . . I	338
Saccoglossus kowalevskii	XP_002730480	68%	982 . . QEL . . SL.K.Y . . ST . . SN.DNQ.E I N . . IL .	1040
Danio rerio	XP_696904	72%	1228 . K . . EL . . L . . L.K.Y . . T.SK . . E . . Q . . V M N . . I	1284
Branchiostoma floridae	XP_002610101	68%	1097 . S.NQL.Q.L . . SY . . R.S . . EKQ.EA I N . . I	1153

Figure 6.3B. Alignment of the putative partial ABCC4, ABCC5-like sequences from *D. magna* with ABC nucleotide binding domains (NBD) of several organisms. Amino acid positions of the comparison ABC sequence stretches that align with the *D. magna* ABC sequences are indicated. The conserved motifs ABC signature and Walker B domain are highlighted in light and dark grey respectively. From all the ABC sequences only amino acids diverging from the *D. magna* ABC sequences are given. Percentage identity is also depicted. Protein codes as those of NCBI.



Figure 6.4A. Phylogenetic tree including the *D. magna* ABCB1 sequences with the first 100 identified sequence hits. Gene definitions are from the GenBank original description, without considering their adequacy to the actual phylogenetic position of the sequence in the tree. Clustering only considered the protein region equivalent to the one sequenced in the *D. magna* abcb1 gene identify as “p glycoprotein partial *Daphnia magna*”.

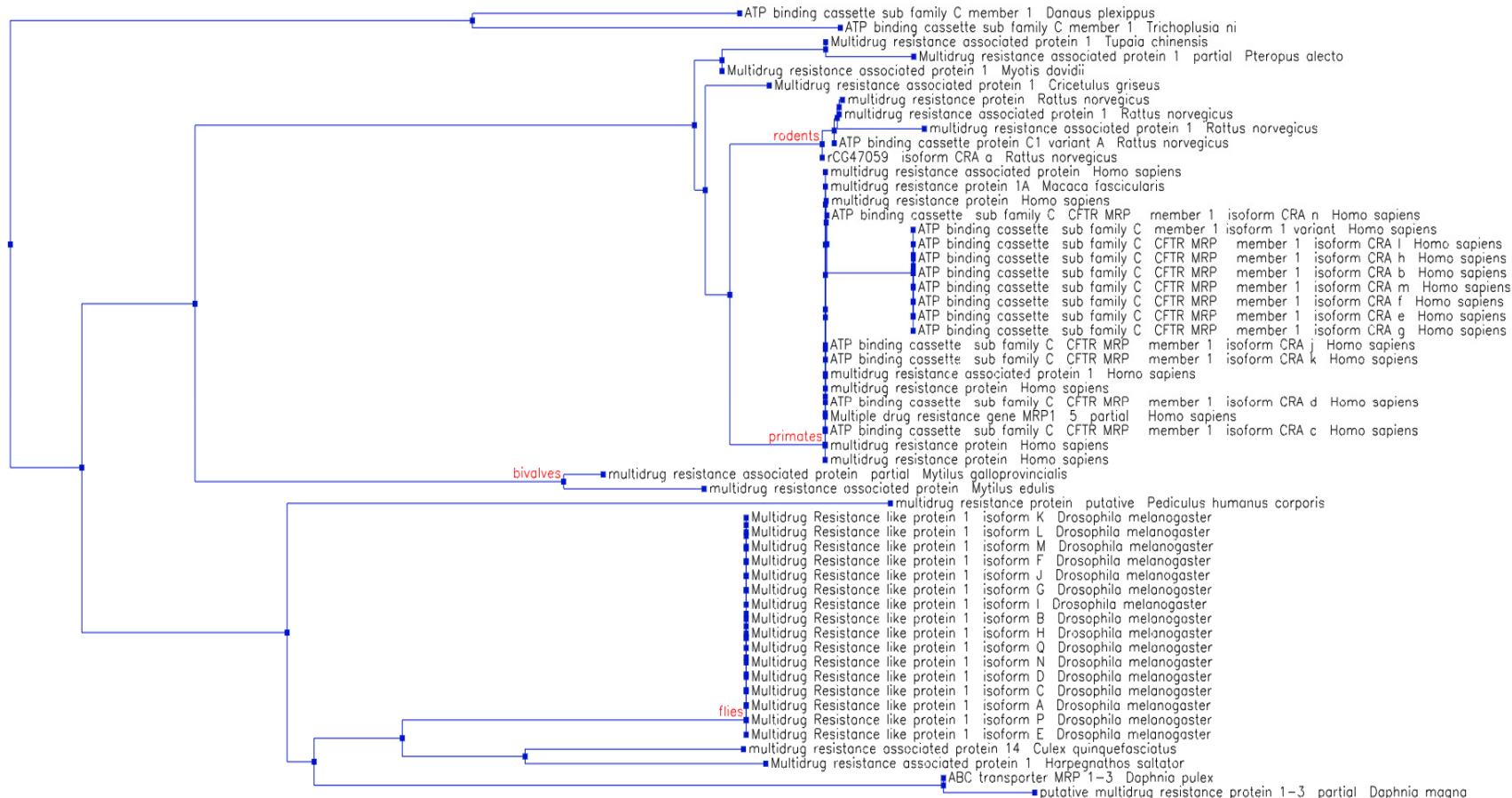


Figure 6.4B. Phylogenetic tree including the *D. magna* ABCC1-3 sequences with the first 100 identified sequence hits. Gene definitions are from the GenBank original description, without considering their adequacy to the actual phylogenetic position of the sequence in the tree. Clustering only considered the protein region equivalent to the one sequenced in the *D. magna* abcc1-3 gene identify as “putative multidrug protein 1-3 partial *Daphnia magna*”.

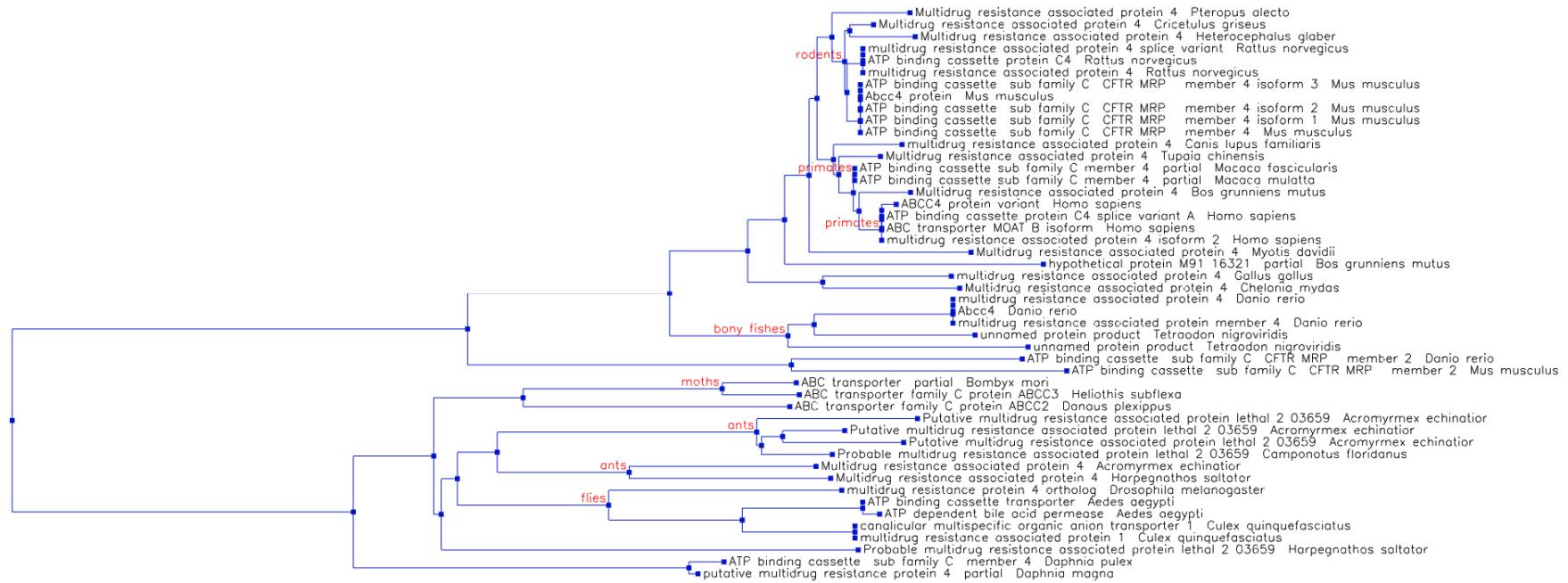


Figure 6.4C. Phylogenetic tree including the *D. magna* ABCC4 sequences with the first 100 identified sequence hits. Gene definitions are from the GenBank original description, without considering their adequacy to the actual phylogenetic position of the sequence in the tree. Clustering only considered the protein region equivalent to the one sequenced in the *D. magna* abcc4 gene identify as “putative multidrug protein 4 partial *Daphnia magna*”.

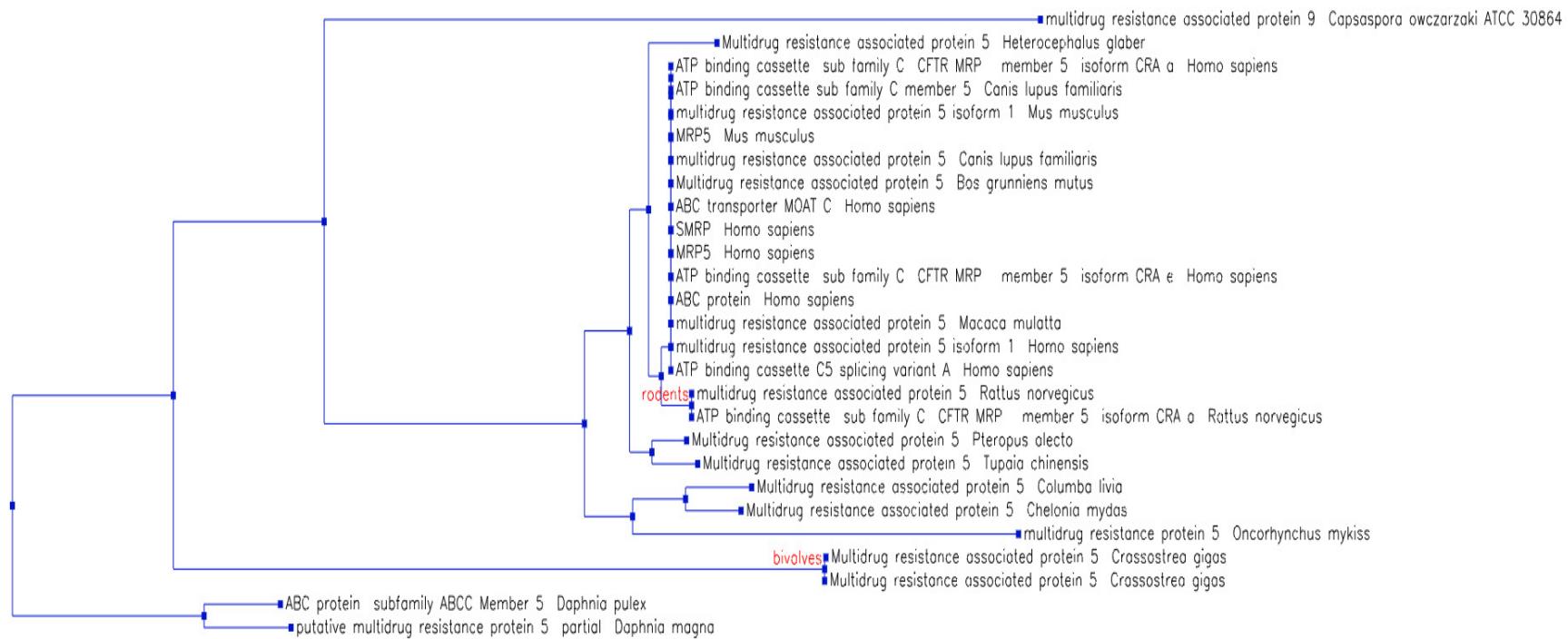


Figure 6.4D. Phylogenetic tree including the *D. magna* ABCC5 sequences with the first 100 identified sequence hits. Gene definitions are from the GenBank original description, without considering their adequacy to the actual phylogenetic position of the sequence in the tree. Clustering only considered the protein region equivalent to the one sequenced in the *D. magna* abcc5 gene identify as “putative multidrug protein 5 partial *Daphnia magna*”.

The aim of this part of the study was to determine whether putatively MXR associated genes are transcribed at all in *D. magna* and our data indicate that this is the case. Since our sequence data were obtained from RNA, these data show that these ABC transporter genes that highly correspond with predicted gene sequences from *D. pulex* (Sturm *et al.*, 2009) are active genes that are indeed transcribed in *D. magna*.

Further, although other physiological functions of the transporter proteins need to be considered transcription activity of *abcb* and *abcc* genes that in other organisms are associated with MXR function can be seen as indication that MXR efflux activity based on the respective transporter proteins may be found in *D. magna*.

The sequences we obtained with PCR are from one representative of each homologue of mammalian *ABCB1*, *ABCC1/ABCC3*, *ABCC4* and *ABCC5*. Among ABCB full transporters one other gene beside the one of which we obtained partial cds from *D. magna* and which in *D. pulex* encodes for protein Dappu-347264 was predicted from *D. pulex* genomic sequences, which encodes for Dappu-347265. Both *D. pulex* proteins, as also the *D. magna* sequence, cannot be clearly associated with any mammalian ABCB full transporter ortholog (Sturm *et al.*, 2009); Fig. 2); the *D. magna* gene is therefore called here *abcb*. We were not able to amplify transcript corresponding to the Dappu-347265 encoding sequence and this gene may therefore not be transcribed in *D. magna*. Within the ABCC sub-family the *abcc1/3* sequence of *D. magna* could not be associated with mammalian *ABCC1* or *ABCC3*. There are similarities in functional properties of mammalian *ABCC1* and *ABCC3*. Both proteins are multidrug resistance associated proteins conferring cellular efflux of conjugated compounds (Deeley and Cole, 2006) and a likewise function may also be assumed for the, on sequence level, comparatively similar *D. magna* *Abcc1/3*.

There are no Abcc transporters in *D. pulex* that could be associated with *ABCC2*, another mammalian multidrug resistance associated protein, but one representative similar to mammalian *ABCC4* and three *ABCC5* like isoforms (Sturm *et al.*, 2009). The *Abcc5* sequence that we identified in *D. magna*

corresponds to the isoform encoding for the EFX72656 protein in *D. pulex* (Fig 6.3B).

Abcb and *abcc1/3* transcript abundances in different life stages of *D. magna* as determined by qPCR were relatively low in eggs and embryos and increased in neonates and juveniles, whereas *abcc4* and *abcc5* transcripts were constantly at comparatively high levels in all life stages (Fig. 6.5A). Across transporters, transcript levels were lowest for *abcb* in eggs and juveniles with levels 10- to 15-fold below *abcb* levels in the older stages. *Abcc1/3* levels that were also comparatibely low in eggs and embryos were about five-fold higher than *abcb* transcript levels in those stages. Compared to the other life stages examined transcript levels of all transporters were highest in juveniles. The different *abcb* and *abcc1/3* transcript levels in different *D. magna* life stages resemble expression patterns of corresponding homologs in other aquatic invertebrate species. In mussels mRNA levels and transporter activity of *abcb1/Abcb1* or/and of *abcc/Abcc* transporters are absent in eggs but they increase dramatically in the one day trocophora larvae (Faria *et al.*, 2011b; McFadzen *et al.*, 2000; Navarro *et al.*, 2011; Navarro *et al.*, 2012b). Function of those ABC transporters in early life stages of aquatic invertebrates has been associated with protection against potentially toxic chemicals dissolved in the water as development takes place outside of a parental organism and in direct contact with the environment (Faria *et al.*, 2011b; Hamdoun *et al.*, 2004; McFadzen *et al.*, 2000; Navarro *et al.*, 2012b; Roepke *et al.*, 2006; Toomey *et al.*, 1996). Although *Daphnia* eggs and embryos develop inside a maternal brood pouch they are also in direct contact with the water and with dissolved chemicals therein. Therefore, it may be assumed that ABC transporters likewise serve as defence against xeno-toxicants from the outside. Further, transporters from the ABCC subfamily may flux out metabolites from cells, another pivotal function for development of embryos (Shipp and Hamdoun, 2012).

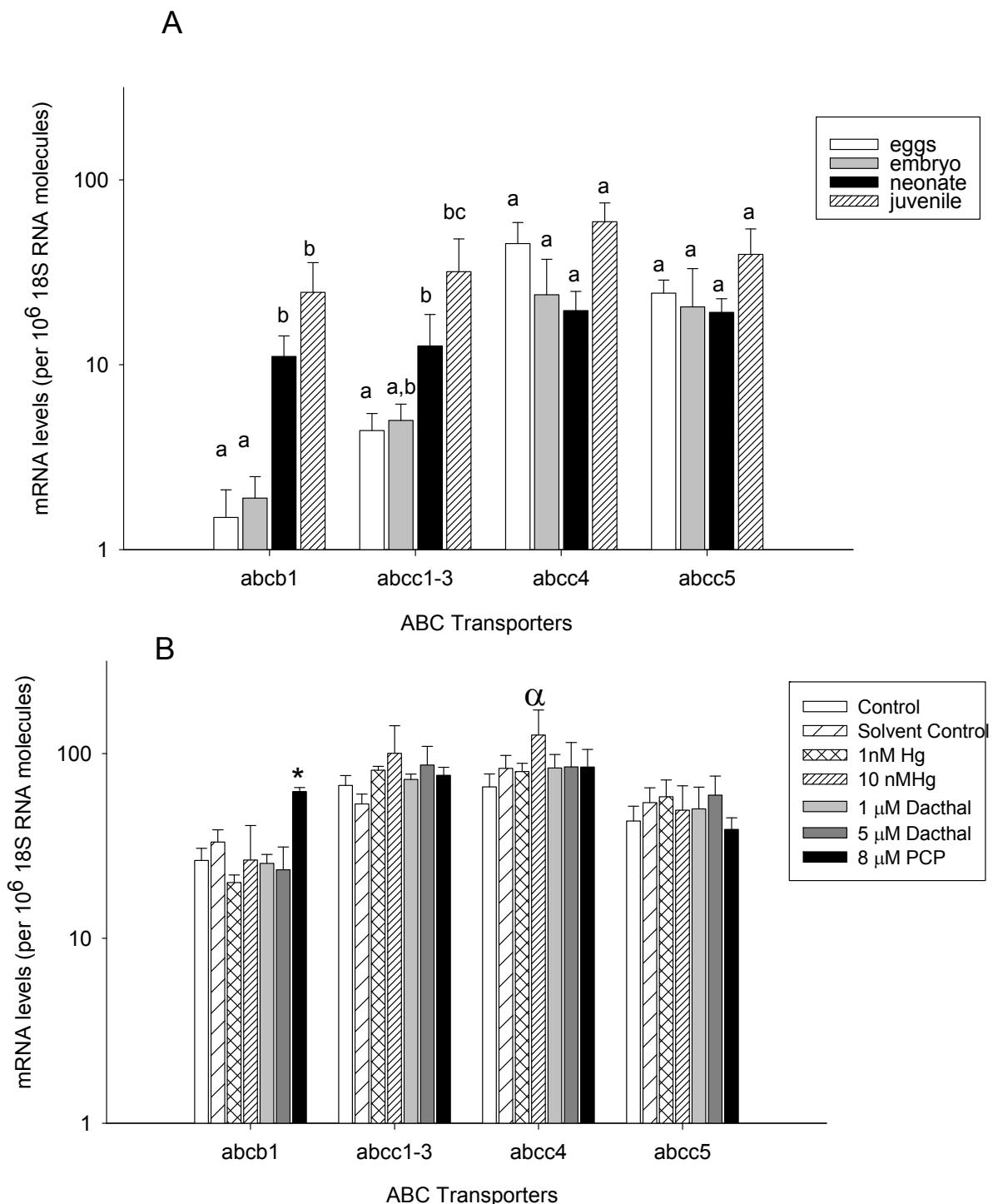


Figure 6.5. Relative levels of putative abcb, abcc1/3, abcc4, abcc5 transporters depicted as relative mRNA abundance of 18S ribosomal (Mean \pm SE, n=5-9) in *D. magna* eggs, embryos, newborn neonates, and juveniles of 4 days (A); and in juveniles of 4 days pre-exposed for 24h to model inducers. Different low case letters in graph A or symbols in graph B indicate significantly ($P<0.05$) different sets of data after ANOVA and Tukey's or Dunnett's posthoc tests, respectively. In graph B mercury (Hg) results have been contrasted with those of control, whereas those of dacthal and pentachlorophenol (PCP) with solvent controls.

Exposure of *D. magna* juveniles to sub-lethal concentrations of inorganic Hg (1, 10nM), dacthal (1, 5 μ M) and pentachlorophenol (5 μ M), which have been shown to induce expression of MXR transporters in the past in mussels (Eufemia and Epel, 2000; Navarro *et al.*, 2012b), had only little effects on transporter transcript levels (Fig 6.5B). *Abcb* transcript levels were significantly elevated only in the pentachlorophenol treatment group and transcript levels of *abcc4* were significantly above controls in the 10 nM Hg treatment ($P<0.05$, based on ANOVA and Dunnett's tests). These data thus show that mRNA expression of *abcb* and *abcc4* transporter genes can be induced by chemical stress. Although it needs to be considered that the applied compounds do not have the same transcription inducing effects of transporter genes in *D. magna* as in other species and/or that the *abcc1/3* and *abcc5* transporter genes are not chemically inducible it is also conceivable that the applied concentrations of the test compounds were not sufficient to induce transporter transcription. This may be the case for dacthal that had a *D. magna* 48h-LC50>150 μ M but not of mercury and pentachlorophenol since the tested concentrations were just two fold lower than the obtained 48h-LC50s (20nM Hg, 16 μ M PCP; results obtained from preliminary experiments). Nevertheless, selected concentrations of inducers were within the range used in previous studies (Eufemia and Epel, 2000; Navarro *et al.*, 2012b)

6.4.3. Dye efflux assays

Ex-vivo assays showed that both ABCB1, ABCC specific and unspecific inhibitors of MXR transporters (REV203, MK571 and CsA, respectively) were able to inhibit transporter activity leading to the accumulation of trace dyes in the organisms in a concentration-dependent manner (Fig. 6.6). The high variability observed, however, across replicates prevented the estimation of significant ($P<0.05$) IC50 in most occasions using regression approaches (Table 6.3).

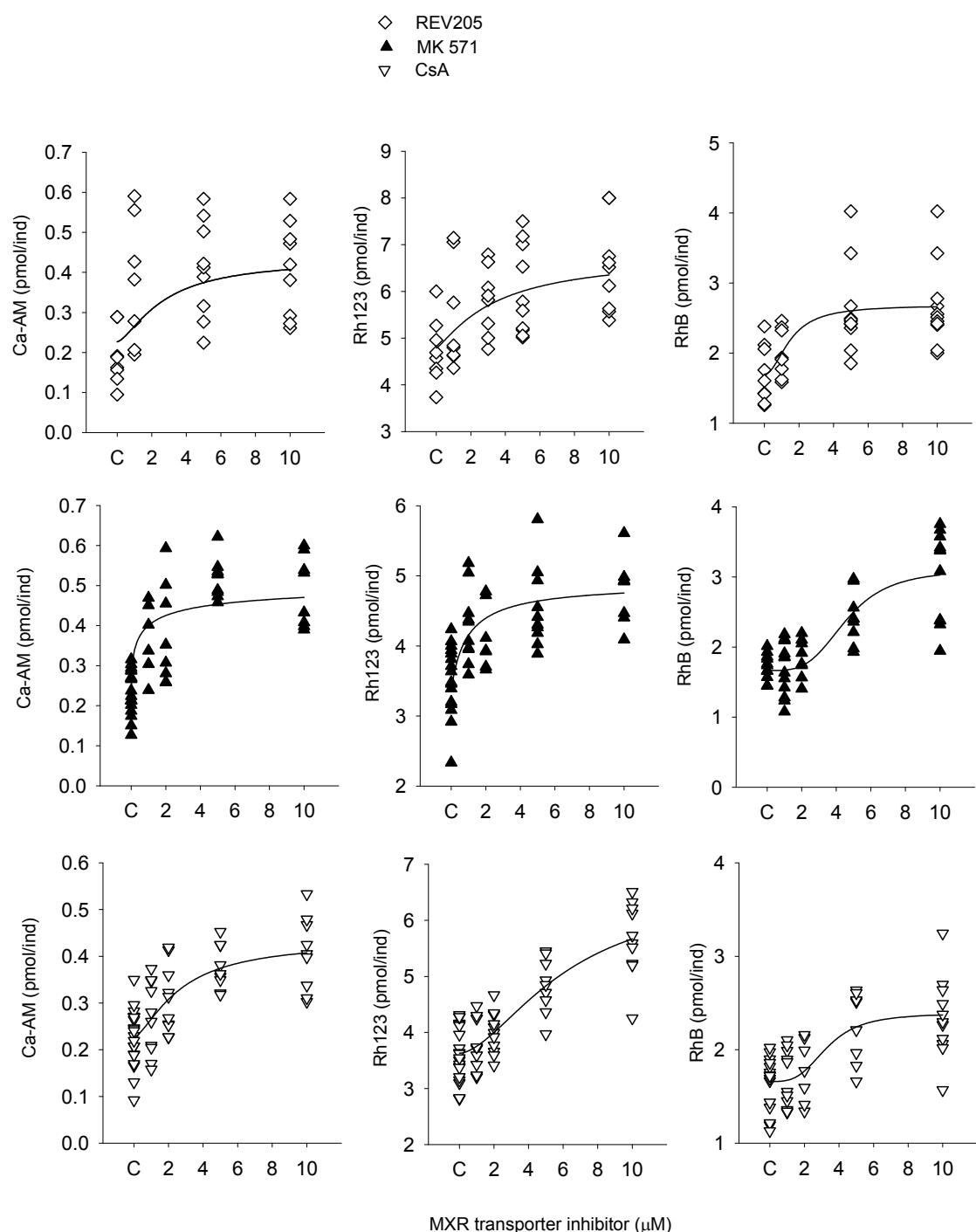


Figure 6.6. Effect of transported MXR inhibitors on the accumulation of Ca-AM, (measured as calcein) (left), Rh123 (middle) and RhB (right graph pannel) (pmol/individual) in *D. magna* juveniles. Symbols are single measurements. Data were fitted to the four parameter dose-response Hill model of eq. 6.1. Solvent controls (C) are also included.

Table 6.3. Inhibition concentration (IC50) values based on nominal concentrations and its standard error (SE), maximal accumulation (Max, fold increase) of Ca-AM, Rh123 and RhB with the model inhibitors used. The lowest significant ($P<0.05$) concentration effect in dye accumulation levels relative to controls (LOEC) is also depicted . N, sample size, r^2 , r square. ns, $P\geq 0.05$: * $0.01 < P < 0.01$; *** $P < 0.001$

Substrate	Parameter	Model Inhibitor		
		REV205	MK571	CsA
Ca-AM	Max	2.2	2.2	1.9
	IC50 (μM)	0.3 (ns)	1.5(0.6)	2.7 (ns)
	LOEC (μM)	1	1	2
	r^2	0.34 **	0.64 ***	0.50 ***
	N	48	60	68
Rh123	Max	1.4	1.5	1.8
	IC50 (μM)	3.4 (ns)	0.9 (ns)	6.2 (ns)
	LOEC (μM)	5	1	5
	r^2	0.26 **	0.51 ***	0.57***
	N	38	59	70
RhB	Max	1.6	1.9	1.4
	IC50 (μM)	1.5 (ns)	4.6 (0.7)	3.1 (1.1)
	LOEC (μM)	5	5	5
	r^2	0.36 ***	0.60 ***	0.42 ***
	N	39	58	60

Nevertheless, ANOVA results depicted as estimated LOEC indicated that in all the transporter inhibition assays there was a significant ($P<0.05$) increase of accumulated dye relative to controls. These results provided the first indication of the activity of ABC transporter activity in *D. magna*.

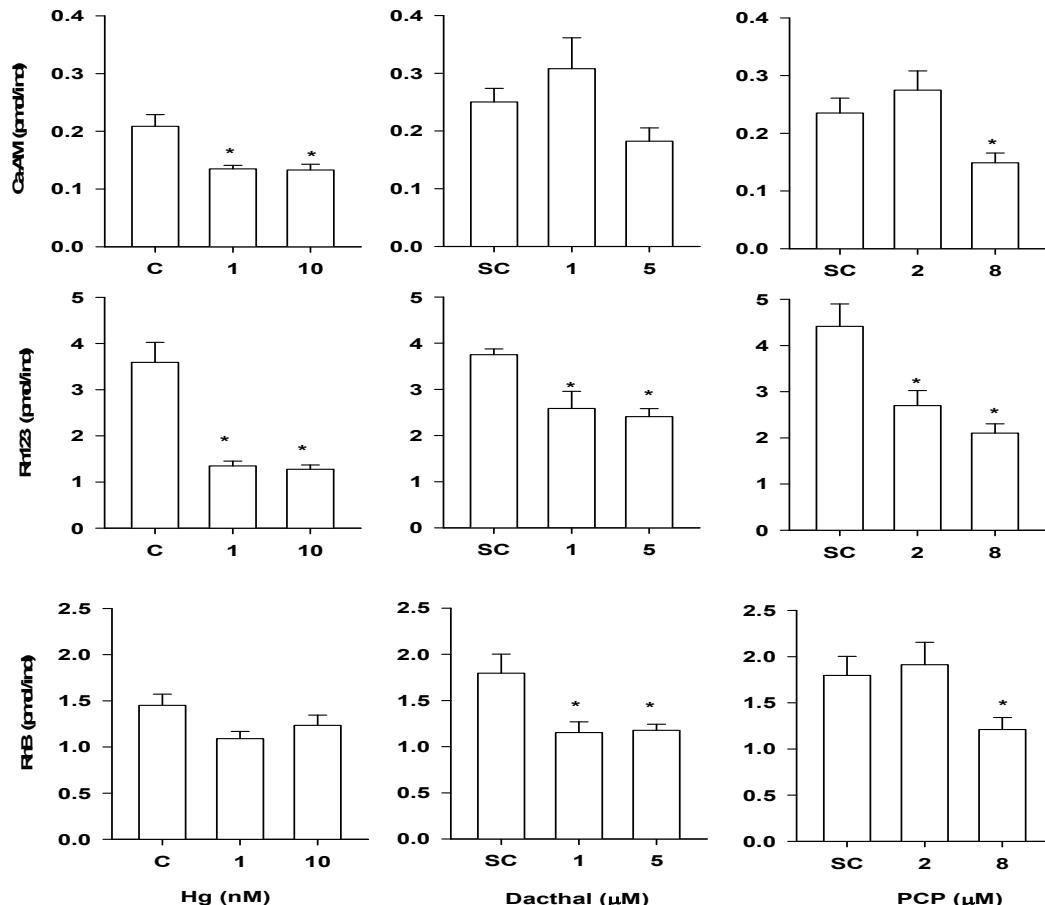


Figure 6.7. Effect of putative transported MXR inducers on the accumulation (Mean±SE, $N=10$) of Ca-AM, (measured as Calcein), Rh123 and RhB in *D. magna* juveniles after 24h of exposure. Respective control (C) or solvent control (SC) treatments are also depicted. *significant ($P<0.05$) differences from controls or solvent controls after ANOVA and Dunnet's post-hoc tests

The use of ex-vivo assays may have underestimated transporter activity, increasing experimental noise since prevented dyes to be accumulated in the gut, which was the organ having greater levels of fluorescence and probably of transporter activity (images of dye target organs are depicted in Fig 6.1). Nevertheless, in most treatments mean IC50s varied across dyes and transporter inhibitors. For Ca-AM, REV205 had a greater specificity (lower IC50,

Table 6.3) than MK571 but an equivalent inhibition potential (similar maximal fold increase in accumulated dye; Table 6.3). For the dye Rh123, MK571 showed greater specificity than REV205 and CsA (lower IC₅₀, Table 6.3). For the dye RhB, REV203 had a greater specificity (i.e., lower IC₅₀ value) than MK571 and CsA accumulating dye. It has been reported that RhB is a specific ABCB1 substrate, whereas Rh123 and Ca-AM may also be substrates for ABCC1 and ABCC2 as well (Daoud *et al.*, 2000; Essodaïgui *et al.*, 1998; Eytan *et al.*, 1997; Hollo *et al.*, 1994; Holló *et al.*, 1996; Yeheskely-Hayon *et al.*, 2009b). Furthermore, REV205 and MK571 are not fully specific inhibitors of ABCB1 and ABCC, respectively, since at higher µM concentrations show a significant cross-inhibitory activity (Haimeur *et al.*, 2004; Sharom *et al.*, 1999b; Zaja *et al.*, 2008). These findings from mammalian/vertebrate models were confirmed in our experiments in *D. magna* as inhibitors of ABCC transporters in mammalian systems (CsA and MK571) also led to an increase in the accumulation of Ca-AM and Rh123 in *D. magna* juveniles. It is noticeable that REV205, but not CsA, (both putative ABCB1 inhibitors) changed their inhibitory properties depending on the substrate, showing the greatest affinity for Ca-AM followed by RhB. MK571 (a ABCC inhibitor) had the greatest potential to inhibit efflux transporter activity of Ca-AM and the lowest to do so for RhB, which agrees with expectations since Ca-AM is considered a more specific substrate of ABCC than RhB (Essodaïgui *et al.*, 1998; Yeheskely-Hayon *et al.*, 2009b). Maximal level of inhibition across MXR substrate dyes and inhibitors (1.4-2.8) were in the lower range of those reported in mussel gills (2-7 fold), zebra mussel embryos or fish cell lines (7-23 fold) (Luckenbach *et al.*, 2008; Luckenbach and Epel, 2005a; Smital *et al.*, 2004) (Faria *et al.*, 2011b; Zaja *et al.*, 2008).

Pre-exposure of *D. magna* juveniles to inorganic mercury, dacthal and pentachlorophenol, decreased significantly ($P<0.05$) the accumulation of the studied dyes in most occasions (Fig 6.7). In only two treatments there were no significant ($P<0.05$) dye accumulation changes relative to controls. These treatments included accumulation of Ca-AM and RhB of juveniles pre-exposed to dacthal and mercury, respectively. Lower accumulation levels of dye can be interpreted as greater MXR type transporter activity. There is reported evidence that the chlorinated aromatic pesticides dacthal and pentachlorophenol induce

abcb1/Abcb1 gene/ protein expression and related efflux of RhB and Ca-AM in the mussels *Mytilus californianus* and *Dreissena polymorpha* (Eufemia and Epel, 2000; Navarro *et al.*, 2012a; Tutundjian and Minier, 2007a). Mercury induced expression of mammalian ABCC1 (Kim *et al.*, 2005) and increased abcc gene expression and Abcc transporter activity in zebra mussel (Navarro *et al.*, 2012a). Ca-AM is known to be specific substrate of ABCB1 and ABCC transporters and RhB and Rh123 a better substrate for ABCB1 and ABCC transporters, respectively (Daoud *et al.*, 2000; Essodaïgui *et al.*, 1998; Eytan *et al.*, 1997; Holló *et al.*, 1994; Holló *et al.*, 1996; Yeheskely-Hayon *et al.*, 2009b). Results obtained for RhB, then, agree with expectation since only dacthal and pentachlorophenol decreased the accumulation of the dye in pre-exposed juveniles. All three inducers decreased the accumulation of Rh123 having mercury the strongest effects. This may indicate that in *D. magna* dacthal and pentachlorophenol also induced Abcc type transporters or that *D. magna* Abcb like transporters were not as specific as mammalian ones for rhodamine dyes, thus showing also high affinity for transporting RhB. The second argument seems to be the most feasible provided the observed lack of specificity of Abcb and Abcc type efflux activity across the studied dyes (Fig 6.6). The lack of effect of dacthal decreasing the fluorescence levels of Ca-AM but not of RhB and Rh123 may be also related to secondary side effects related with the hydrolysis of Ca-AM by cellular esterases.

The results obtained from induction experiments (Fig 6.5B and Fig 6.6) could be summarized as follows: Under exposure to mercury, increased levels of mRNA of abcc4 (Fig 6.5 B) were related with low rates of accumulation of Ca-AM and of Rh123 but not of RhB (Fig 6.7), which may indicate that mercury is inducing functional abcc4 like transporters. In fish cell lines and zebra mussel gills mercury induces mRNA levels of abcc1-4 transporters and their related transporter activity (Della Torre *et al.*, 2012; Navarro *et al.*, 2012b). Dacthal induction results were not conclusive since this pesticide decreased the accumulation of RhB but not of Ca-AM, despite that both compounds are substrates of ABCB1 transporters; decreased the accumulation of a putative ABCC transporter (Rh123) and did not affect transcription levels of the studied abc genes. In zebra mussel embryos and adult gills dacthal was also not a specific inducer of Abcb1 transporters since it also induced abcc genes and

the putative transporter activity (Navarro *et al.*, 2012b). This means that dacthal may be detoxify by other MXR transporters than the ones sequenced in the present study, which is likely to occur provided the high diversity of abc transporters found in the genome of *D. pulex* (Sturm *et al.*, 2009). Results for pentachlorophenol also agree with those obtained in *M. californianus* (Eufemia and Epel, 2000) since this compound increased mRNA transcripts of abcb1 and also the associated transporter activity of RhB and Ca-AM.

In summary induction experiments provided further evidence that the sequenced abcb1 and abcc4 *D. magna* genes, that were homologous to those of *D. pulex* (Sturm *et al.*, 2009) are likely to be ABCB1 and ABCC transporters, whereas the functionality of the remaining gene sequences is still unknown.

6.4.4. Toxicity responses

Toxicity concentration-response curves for all tested chemicals in *D. magna* juveniles at 24 and 4 h are shown in Fig. 6.8. In the case of CsA, its maximum water solubility levels (100µM) corresponded to the LC30 value at 24h. As expected, toxicity (LC50, Table 6.4) increased with exposure time being the greatest effect observed for CsA and mitoxantrone. It is important to note that toxicity of chlorambucil hardly increased with exposure time, probably because it's relatively low stability in ASTM water. Estimated regression parameters of eq. 2 showed a good model fit ($r^2 \geq 0.78$; Table 6.4) of all single concentration-response curves.

Table 6. 4. Regression parameters according to eq. 2 of fitted models to single exposures of the studied chemicals. All regressions were significant ($P < 0.001$). N, sample size, r^2 , r square. LC50 are based on nominal concentrations.

Compound	Exposure time (h)	LC50 (µM)	95% CI (µM)	p	r^2	N
Chlorambucil	24	102	95.2	108.8	5.3	0.87
	48	77.4	75.4	79.4	15.9	0.93
Mitoxantrone	24	45.7	37.7	53.7	2.3	0.84
	48	11.6	9.5	13.7	2.4	0.94
REV205	24	35.7	32.6	38.8	2.2	0.94
	48	15.1	13.9	16.3	3	0.97
MK571	24	89	86.4	91.6	6.6	0.91
	48	80.3	75.3	85.4	5.7	0.78
CsA	24	160.1	115.9	204.3	1.6	0.79
	48	24.6	20.4	28.8	2	0.88

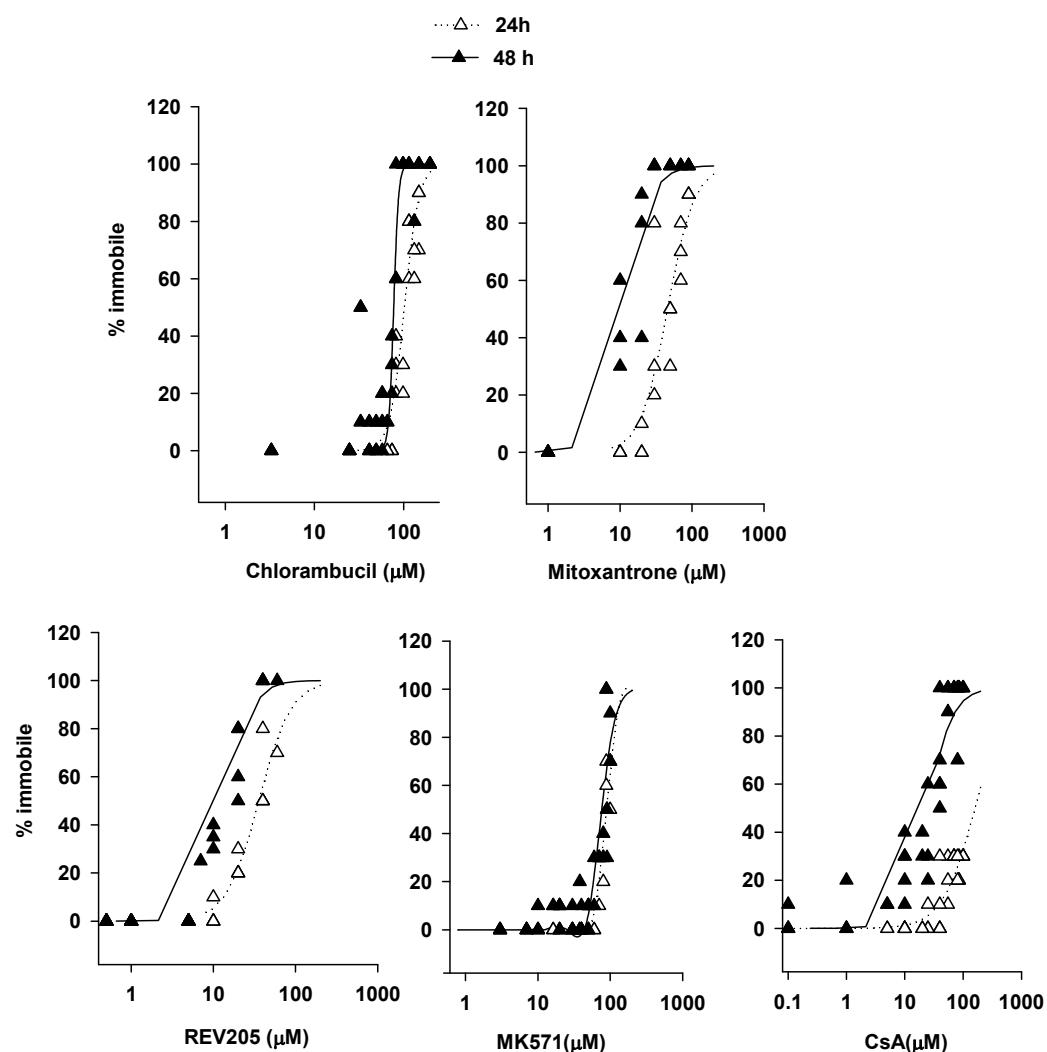


Figure 6.8. Percentage of immobile *D. magna* juveniles after 24 and 48h of single exposure to the studied compounds. Each symbol corresponds to a single replicate. Responses have been fitted to the Hill regression model of eq. 2 (see text). Horizontal axis is depicted in log scale. Open and black triangles or doted and strike lines correspond to 24 and 48h exposures, respectively. Triangles are single observations

The mode of action of the studied chemicals is unknown in *D. magna* but those of cytostatic drugs are well known in mammalian cells and used to treat cancer. Chlorambucil is an anti-neoplastic agent used to treat lymphocyte leukaemia that belongs to the group of alkylating agents (Barnouin *et al.*, 1998). The human multidrug resistance protein (ABCC1) confers resistance of cells to chlorambucil (Barnouin *et al.*, 1998). Mitoxantrone, a ABCB1 substrate, belongs to the series of anthraquinones and stop tumour growth by cross-linking guanine bases in DNA double-helix strands - directly attacking DNA (Kizek *et al.*, 2012; Shen *et al.*, 2009). There is also evidence that CsA is an inhibitor of different cytochrome P450 Cyp enzymes in humans, and MK571 is a leukotriene receptor agonist (Lin and Lu, 1998; Mellor *et al.*, 2001; Moothala and Renton, 1986). Considering that it is likely that the five studied compounds act through different mechanisms, we should expect *a priori* joint additive effects to be predicted by the IA concept. Nevertheless, the alternative argument that the tested chemicals could act by the same mechanisms of action cannot be discarded. Therefore, the observed joint effects were confronted with effects predicted by IA and CA concepts.

From the 19 binary mixtures and exposure period combinations from which it was possible to obtain defined concentration-response joint effects (Fig. 6.9), those involving the studied cytostatic compounds and the model inhibitors MK571 and CsA and CsA and MK571 (9 mixtures) showed toxic effects greater than additivity according to both IA and CA concepts (Table 6.5). Synergism in terms of fold changes in toxicity relative to reference additive models (i.e. IA) was as high as 7.4 fold for the binary mixture of mitoxantrone and CsA. Binary combinations involving the studied model inhibitors and chlorambucil showed greater synergistic effects at 24 than at 48 h probably since chlorambucil disappeared from water shortly after exposure. Consequently, chemosensitization effects of the studied MXR inhibitors impairing the efflux of this drug were greatest during the first hours of exposure. Alternatively, synergistic effects of CsA were greater at 48 h than at 24 h. We consider that this retarded effect of CsA may be related to its high molecular weight and hence to its lower bio-availability to *D. magna* juveniles. Binary combinations of cytostatic drugs with REV205 only showed moderate levels of enhance toxicity

(1.5 fold) relative to IA predictions for chlorambucil at 24 h being antagonistic or additive in the remaining seven studied pair combinations. These results disagree with expectations since chlorambucil is known to be effluxed out by ABCC transporters in mammalian cells and REV205 is a known ABCB1 inhibitor. Similarly greater than additive toxic effects may also be expected for pairings of inhibitors targeting the same transporter type (REV205 with CsA). Nevertheless, it is important to consider that in *in vivo* assays REV205 showed a great potential to inhibit filtering rates of *D. magna* juveniles decreasing the uptake of Rh123 that was used as a surrogate substrate of MXR transporters (see Fig S4, Supplementary Information). Thus, it is likely that REV205 prevented both the uptake and the release of the studied drugs or inhibitors. This means that the inhibitory effect of REV205 on MXR transporters, which would increase internal cell concentrations of cytostatic drugs or CsA, may have been overcome by the inhibition of filtering rates and the consequent reduction of drug uptake. Joint toxicity of binary combinations of cytostatic drugs or between inhibitors targeting different transporters (REV205 and MK751) should be expected to be additive and accurately predicted by the IA concept. Results reported in Fig 6.9 and those depicted in Table 6.5 agree with expectations. Joint synergic effects greater than IA and CA predictions have been seldom reported in mixture toxicity evaluations conducted with individuals (Altenburger *et al.*, 2003). This means that our success in getting synergic effects (5 out of 8 binary combinations) is uncommon and support the statement that chemosensitization increased the joint toxicity of chemical mixtures to a greater extent than predicted by IA and CA concepts probably by favouring the accumulation of cytostatic drugs or inhibitors as it was shown indirectly in the dye efflux assays. Obviously mixture toxicity results are not conclusive and cannot discard alternative mechanisms of action that may enhance toxicity more than expected. For example CsA, which is also known to inhibit P450 enzymes in humans (Moochhala and Renton, 1986) may also inhibited them in *D. magna* resulting in a reducing metabolism of cyclostatics. In our study CsA was the chemosensitizer showing greater synergic effects when co-exposed with the studied cytostatics, an effect that could be explained due to the fact that CsA inhibits the two transporter subfamilies (ABCB, ABCC) or due to the joint

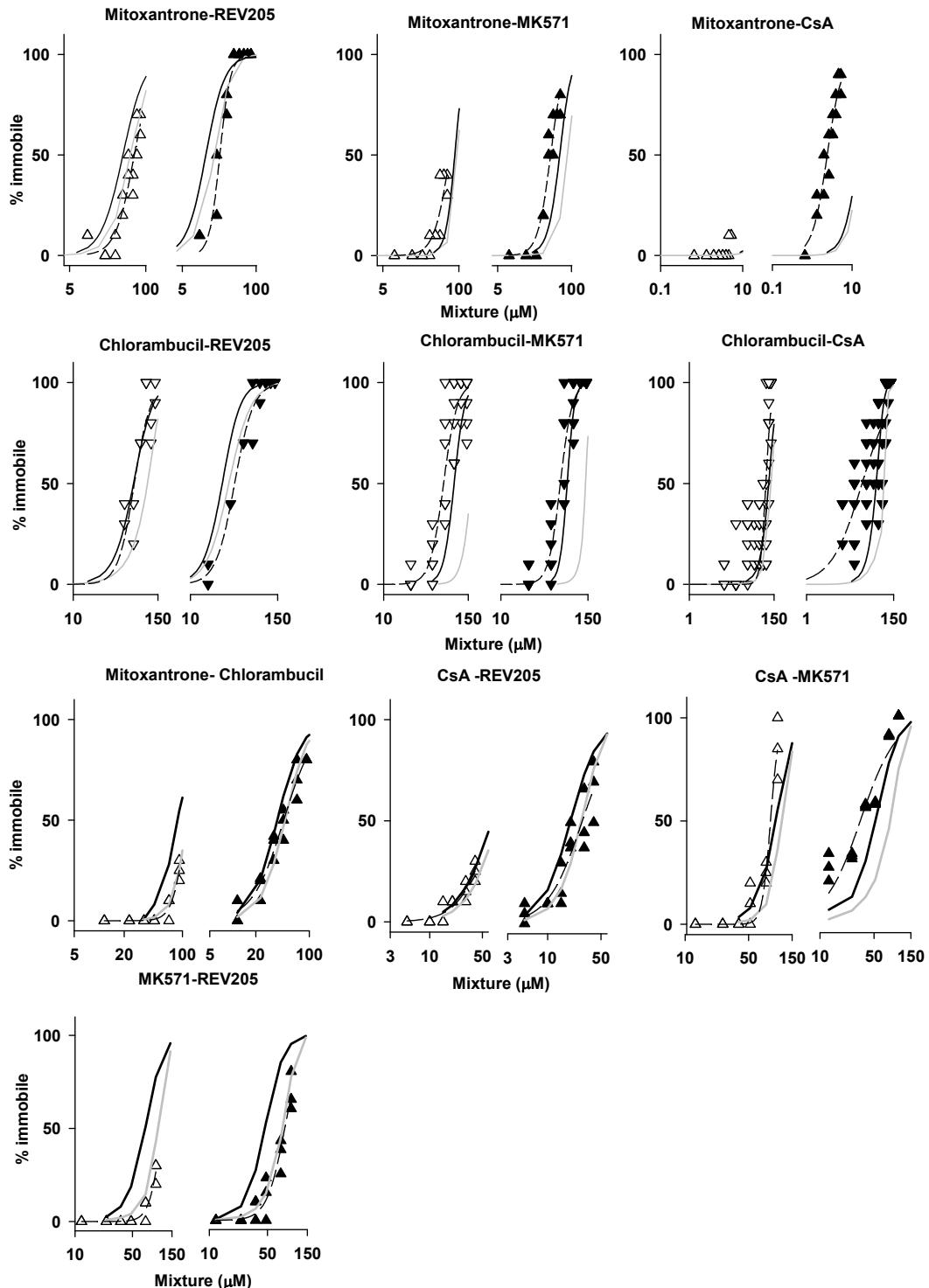


Figure 6.9. Percentage of immobile *D. magna* juveniles after 24 and 48h of exposures to equitoxic binary mixtures of mitoxantrone and chlorambucil with REV205, MK571 and CsA. Each symbol corresponds to a single replicate. Concentration – responses curves predicted by the CA (black lines) and IA (grey lines) concepts are also depicted. Fitted Hill curves (eq. 6.2) to observed joint effects are depicted as short dashed lines. Horizontal axis is depicted in log scale. Within each binary combination left and right graphs or open and black triangles correspond to 24 and 48h exposures, respectively.

Table 6. 5. Summary of mixture assays showing estimated median lethal concentration effects referred to nominal concentrations; LC50s (95% CI) of observed joint responses according to the model of eq 2 and those derived from the IA and CA concepts (eqs 3,5). Fold changes of median lethal effects due to antagonism (clear grey) and synergism (dark grey) are also shown. White coloured cells means that joint effects did not deviate from additivity since 95% CI overlap with mean predictive values according to either IA and CA.

		Time	LC50 (95% CL)	LC50		Fold change	
				IA	CA	IA	CA
Mitoxantrone	REV205	24	63.9 (60.5-67.3)	55.6	40.7	1.1	1.6
		48	23.0(21.1-24.9)	18.2	13	1.3	1.8
	MK571	24	70.1 (58.9-81.3)	93.7	84.9	1.3	1.2
		48	42.9 (39.9-45.9)	82.2	61.9	1.9	1.4
	CsA	48	2.4 (2.2-2.6)	17.8	14.6	7.4	6.1
Chlorambucil	REV205	24	70.9 (60.1-81.8)	107.8	68.9	1.5	1
		48	39.7 (36.2-43.2)	33.1	26.9	1.2	1.5
	MK571	24	68.4 (64.0-72.8)	164.8	95.6	2.4	1.4
		48	64.0(57.6-70.4)	139.5	78.8	2.2	1.2
	CsA	24	94.6 (86.6-102.6)	123.8	109.7	1.3	1.2
		48	26.6 (19.8-33.4)	89.7	55	3.4	2.1
Mitoxantrone	Chlorambucil	24	112.3 (105.1-119.5)	113.4	87.4	1	1.3
		48	46.5 (43.9-49.2)	47.7	38.3	1	1.2
CsA	REV205	24	68.4 (55.6-81.2)	79.7	67	1.2	1
		48	28.0 (24.9-31.2)	26.7	19.6	1	1.4
	MK571	24	87.1 (84.3-90.0)	113.3	97.8	1.3	1.2
		48	34.0 (30.2-37.8)	78.1	51.8	2.3	1.5
REV205	MK571	24	114.4 (101.3-127.5)	102.4	70.4	1.1	1.6
		48	81.1 (72.3-89.8)	74.1	46.5	1.1	1.5

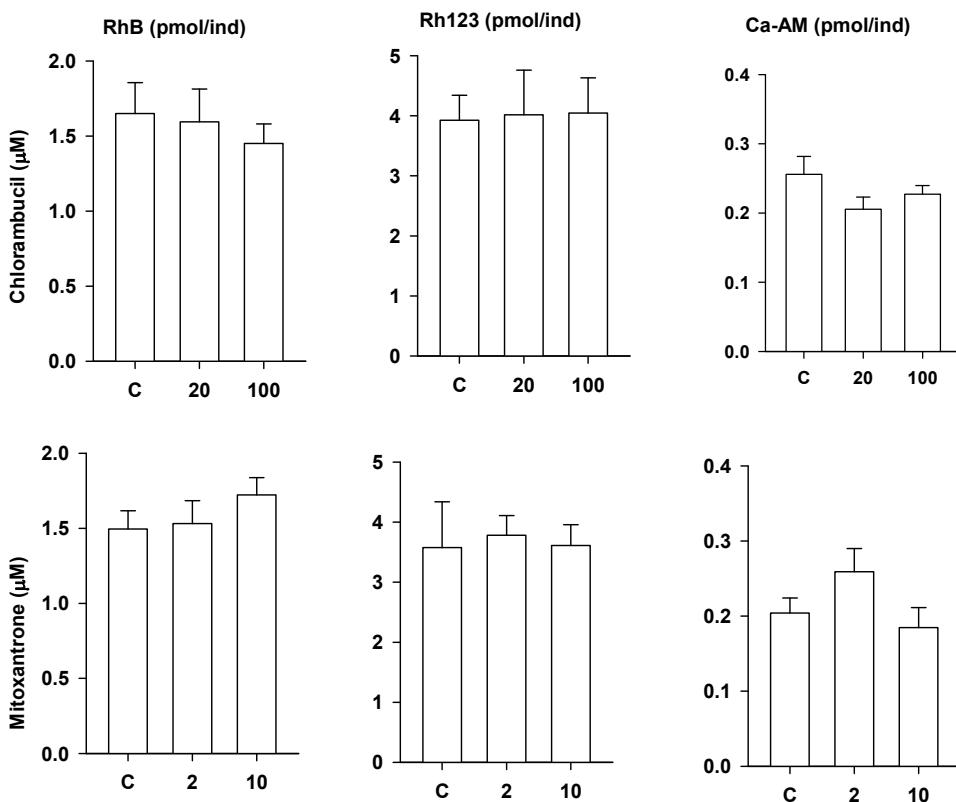


Figure 6.10. Effect of chlorambucil and mitoxantrone on the accumulation (Mean \pm SE, N=10) of Ca-AM, (measured as Calcein), Rh123 and RhB in *D. magna* juveniles. Any of the treatments affected significantly ($P<0.05$, based on ANOVA) the accumulation of the studied dyes.

effects of chemosensitization and metabolism impairment. Other mechanisms of actions such as inhibitory effects on MXR transporters of the studied cytostatic drugs was discarded provided that those substances did not affected dye accumulation (Fig 6.10) and the joint toxicity of their binary mixture was additive (Fig 6.9, Table 6.5). Furthermore, alternative toxic mechanisms of action of the studied competitive inhibitors REV205 and MK571 were also unlikely to occur since the joint toxicity of their binary mixture was also additive (Fig 6.9, Table 6.5).

6.5. Conclusions

In summary our results provide the first evidence for the involvement of the MXR defensive system in toxicological responses of *D. magna* juveniles exposed to mixtures of known chemosensitizers and MXR toxic substrates as follows:

(1) We showed that *D. magna* had gene sequences homologous to previously characterized *D. pulex* abcb1, abcc transporters and that these genes were differentially transcribed across developmental stages showing the greater mRNA levels in fully developed juveniles.

(2) Dye efflux assays indicated that accumulated fluorescence MXR substrates of ABCB and ABCC pumps increased in a concentration related manner in *D. magna* juveniles co-exposed to model ABCB and ABCC inhibitors. These results provide evidence that ABCB and ABCC type transporter activity exist in *D. magna*.

(3) Induction experiments indicate that at least two of the sequenced genes abcb1 and abcc4 were MXT transporters since their transcripts and putative dye efflux activity were induced by pentachlorophenol and mercury, respectively.

(4) Mixture toxicity assays tested the hypothesis that the studied chemosensitizers will inhibit the efflux of the tested toxic cyclostatics also known to be substrates of ABCB and ABCC transporters, increasing cytostatic internal cell concentration and hence toxic effects in whole *D. magna* individuals. Reported results indicate more than additive joint effects for most of the binary combinations involving a MXR model inhibitor and a cytostatic, which support our hypothesis.

These results provide a physiological basis to explain synergism in mixture toxicity evaluations. Such synergistic effects, albeit rarely assessed in natural systems, are unpredictable and hence may have dramatic consequences in environmental risk assessment of chemical mixtures. *D. magna* acute tests are one of the most used toxic assays in aquatic toxicology. Thus the proposed experimental framework may be used to screen for potential chemosensitizers that may have more than additive effects when co-occurring with other pollutants.

6.6. Acknowledgements

This study was supported by the bi-lateral German (DAAD PPP) and Spanish project DE2009-0089, by the Spanish MICINN grants (CGL2008-01898/BOS and CTM2011-439 30471-C02-01) and by FEDER funds

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CHAPTER 7

General Discussion and Conclusions

7.1. General discussion

In the last decades, the scientific community has focused on the evaluation of the toxicity of persistent and widely distributed chemical pollutants (Daughton and Ternes, 1999). Special regulations have been issued for those considered as particularly noxious for the environment, with the ultimate target of their abolishment whenever possible, or, at least, a severe reduction on their production and use (Barceló, 2003). The recent development of new and more powerful analytical methods have allowed the detection of new forms of contamination previously considered of no environmental relevance (Barceló, 2003). The study of these so-called emergent pollutants/effects is included in the priority research guidelines of many environmental- and health-regulation bodies, such as the World Health Organization (WHO), the USA Environmental Protection Agency (EPA) or the European Commission (EC). One of the properties of emerging pollutants is their low acute toxicity but their potential sub-lethal effects and toxicological mode of action is unknown or only known in humans (Fent et al., 2006). Furthermore, many emerging pollutants have a common Mode of Action across species but their final effects may vary dramatically across them. For these reasons their environmental diagnosis requires the development of new assessment tools and mostly new conceptual toxicological approaches.

Emerging pollutants effects can be very diverse. An important effect can be the impairment of physiological responses of live organisms through changes of several molecular mechanisms. Important ones include nuclear receptors, metabolism, oxidative stress and chemosensitization effects (Bolong et al., 2009; Epel, 1998; Farré et al., 2008). Emerging chemicals may also act specifically in some key developmental processes, as proliferation, apoptosis, or differentiation, as well as to impair some specific responses of adult stages such as growth and reproduction and behavioural responses related to the ecological fitness (swimming, foraging, escaping reactions) (Farré et al., 2008) but also changing the environment itself.

Emerging effects may also be measured beyond individuals at the population and community levels using laboratory and field assays. Environmental features such as exposure to pollutant mixtures and changes in the food supply are likely

to increase due to global change (chemicals usage increase and/or climate change). Thus these particular aspects need to be addressed in future investigations. Besides toxicological studies, new advances in physiology have underline the importance of impairment of the neuro-endocrine system of aquatic organisms and the importance of natural factors such as food supply.

In this thesis I have focused in three different types of emerging effects that have seldom been considered until now. In chapters two, three and four I presented data showing that environmentally relevant levels of SSRIs and 4-nonylphenol are able to increase offspring production in *D. magna* in a non-adaptive way. 4-Nonylphenol increase offspring production but reduced offspring size irrespectively of food ration levels. This means that females exposed to 4-nonylphenol will produce maladaptive juveniles (i.e. juveniles will be smaller than the optimal size for that environment). According to life-history theory (Sibly and Calow, 1986) *D. magna* females adjust the size of their offspring to maximize its success rate under a particular natural environment, namely the food availability conditions. The effects of 4-nonylphenol can be viewed as maladaptive in the sense that mothers will produce smaller offspring than their evolutionary optimal size for a given food and maternal environment (Barata and Baird, 1998). There is also studies showing that smaller offsprings are also more sensitive to changes, environmental and chemical (Naylor et al., 1992)

Detrimental effects of SSRIs were not obvious in chapter 2 since juveniles exposed to SSRIs matured earlier and produce more offspring of apparently similar size than unexposed individuals. Reproducing earlier and producing more offspring of the same quality means that those females will have a higher fitness (intrinsic rate of growth of the population) (Campos et al., 2012b). According to the principle of energy allocation, every change in life-history carries, as backdrop, a negative effect (Sibly and Calow, 1986). In my exposure scenario, I was able to observe an increase in the reproductive output of both SSRI and 4-nonylphenol, but there was only a negative trade-off (smaller offspring) in the 4-nonylphenol exposure. This observation apparently contradicted life-history theory on fitness costs. Because of this, I proceeded to study interactive effects of SSRIs and food ration levels. Growth and

reproduction in *Daphnia* is tightly tuned across food ration levels such that female adults adjust their *per offspring* reproductive investment to maximize offspring fitness across food environments as follows: at high food ration levels adult females produce more but smaller offspring and the opposite trend occurs at low food levels (Barata and Baird, 1998). *Daphnia* juveniles also grow faster at high food levels and consequently matured earlier than at low food ration levels (Barata and Baird, 1998). *D. magna* life history responses across food ration levels and SSRs indicated that the latter compounds changed the *Daphnia* perception of food availability switching this perception towards higher food ration levels. That is, at limiting food ration levels *Daphnia* individuals exposed to SSRIs matured earlier and produce more but smaller offspring than non-exposed individuals. This switch *per se* is maladaptive since non optimal *per offspring* reproductive investment will translate into lower offspring fitness. Nevertheless, it was still intriguing to understand how animals exposed to SSRIs were able to invest more resources into growth and reproduction without any costs for example on survival. To solve this query in chapter 3 I studied the mode of action of SSRIs assessing if they acted like in humans altering the serotonergic signalling pathways as well as if SSRIs had any physiological costs (Campos et al., 2012a). Observed increases in total offspring production under SSRIs did not occur when animals were also co-exposed to the serotonin receptor antagonist (ciproheptadine), thus indicating that SSRIs in *Daphnia* had a similar target than in humans (increased serotonin activity). Feeding, respirometry and biochemical assays indicated that *Daphnia* individuals exposed to SSRIs, despite of eating the same amount of food, consumed more oxygen irrespectively of food and also had lower carbohydrate reserves than non-exposed animals. These results may indicate that SSRIs enhanced aerobic catabolism. In natural environment *Daphnia* populations performs a diel vertical migration cycle (Ebert, 2005) feeding on algae at surface level at night and resting in deep waters during the day. This cycle is an evolutive outcome to cope with fish predation but requires tolerance to the low oxygen levels of deep waters. *Daphnia* species are adapted to the migration across the water column, switching from aerobic to anaerobic metabolism, and hence making them able to tolerate low oxygen conditions of the hypolimnion during daylight. I hypothesise that SSRIs just switched *Daphnia* metabolism to be more aerobic,

which is much more energetically efficient than the anaerobic one. Being more efficient, allowed organisms exposed to SSRIs to grow faster and reproduce more than non-exposed one. Nevertheless, having an aerobic metabolism and low carbohydrate reserves can be costly under low oxygen levels. Survival assays performed under low oxygen levels confirmed the previous arguments since exposed animals to SSRIs survived less than non-exposed ones. This last result is ecologically maladaptive since SSRIs will decreased the tolerance of *Daphnia* populations to anoxia and hence will select out genotypes that migrate less (Ebert, 2005). In chapter 4, I studied transcriptomic responses of *Daphnia* individuals exposed to 4-nonylphenol and SSRIs to try to unravel the molecular mechanisms of action of these compounds enhancing offspring production or increasing growth rates (just for SSRIs). In this study by relating differentially de-regulated gene signalling pathways with phenotypic effects it was possible to establish the molecular mode of action of the studied compounds. To do so, the study benefit from using the so far most advanced and complete custom made *D. magna* microarray (Garcia-Reyero et al., 2012), and Partial Least Square analyses coupled with Selectivity Ratio (PLS-SR) (Dom et al., 2011), which allowed us to related gene expression patterns with phenotypic effects. The results of chapter 4 indicated that SSRIs were affecting the serotonin signalling pathway in a similar way as in humans but resulting in different outcomes. SSRIs de-regulate the neuroendocrine signalling pathway of serotonin but also changed the gene signalling pathways responsible for the regulation of developmental processes, structural constituents and morphogenesis. De-regulation of genes involved in localization, nervous system development and synaptic vesicle transport were also present. In this study I tried also to use new tools, which had the potential to allow a better insight into the molecular effects and how the de-regulated genes relate to each other and which physiological responses are affected by. For that I use KEGG (Kyoto Encyclopedia of Genes and Genomes), an unavailable tool in *D. magna*, but quite well developed in *Drosophila melanogaster*, and can be a good *proxy* to what might be happening in *Daphnia*. When looking to the metabolic pathway maps of *Drosophila* and using our *D. magna* genes, I was able to observe that most of the de-regulated metabolic genes affected by SSRIs belonged to specific regulatory pathways linked with the target pathway of serotonin metabolism, carbohydrate

metabolism and Krebs cycle (Campos et al., 2013a). This supports our working hypothesis that SSRIs can act as neuronal disruptors in *D. magna*. Conversely, 4-nonylphenol altered few transcriptomic signalling pathways. Most of the de-regulated genes by 4-nonylphenol were related to ecdysone, which agree with previous findings (LeBlanc et al., 2000), but were also related with carbohydrate metabolism and Krebs cycle (Campos et al., 2013a). Unfortunately, I did not measure the energy reserves, neither oxygen consumption of 4-nonylphenol exposed animals, so I cannot fully explain the genomic changes. Further work would be needed to better discriminate the Mode-of-Action of 4-nonylphenol.

The findings of these three studies come to highlight the need of better understanding of what are hormetic effects and which are the affected pathways through which the effect is triggered. These results come to support that hormetic responses should be more considered in ecotoxicological evaluations (Calabrese, 2008) and should be addressed in future investigations of emerging effects of drugs, pharmaceuticals and other molecules (Fent et al., 2006).

In chapter 5 I studied toxicological effects of several manufactured nanoparticles (NM) of titanium dioxide different in phase composition but not in size (20 nm) in *D. magna*. Several reviews published on NM testing are claiming new toxicity procedures due to their unique physical-chemical characteristics, namely their size and aggregation capacity (Baun et al., 2008; Handy et al., 2012). Presently NM are tested on aquatic organism following standard protocols (OECD, 1981). The problem with this approach is that most of the exposure scenarios and analysis deal with NM like if they are dissolved substances. This is not a representative scenario, since NM are not soluble, but behave as a particle in the water column, and as many particles, namely silt particles, they can sorb to edible particles making them to settle faster, thus reducing the food availability (Li et al., 2011). This emerging effect seldom has been considered in NM toxicity investigations. By increasing settling rates of algae growing in the water column, NM may deplete food levels for grazers that feed on algae resulting in major effects on the food web. In this chapter I showed precisely that NM of TiO₂ adhere to algae forming large aggregates. Consequently algae-NM aggregates settled faster and hence *D. magna* feeding

on this medium was starving. This means that NM indirectly affected *D. magna* life history traits such as growth, reproduction and population growth rates (Campos et al., 2013b). The novelty of this study was the implementation of conventional 21 days reproduction tests performed with vessels holding larger water columns. This was crucial to allowing me to detect effects mediated by increasing sedimentation rates of algae that in small vessels are undetectable. This study can be important to help clarify the mode of action of NM on organisms. This effect would add with the recently described “Trojan horse” effect (Limbach et al., 2007), an effect in which the NM serve as a transport vehicle of substances (pharmaceuticals, but also toxics) to tissues and cells both by encapsulating them and/or by attaching ions to its surface and opening the door to the interior of cells. These two emerging mechanisms of action of NM should be considered in future studies.

In chapter 6 I tried to characterize the Multixenobiotic Resistance Mechanisms (MXR) system in *D. magna*. MXR is a general detoxification mechanism shared by most organisms. It was first described in cancer cells that became tolerant to chemotherapeutic treatments (Dano, 1973). It includes trans-membrane proteins that act as active pumps transporting out xenobiotics and metabolites out of cells (Dean and Allikmets, 2001). Relatively recently MXR systems have been studied in other organisms, namely aquatic organisms (Epel, 1998). Several studies have hypothesized that many emerging contaminants (musks, PFOS) and traditional ones (organochlorin compounds) may compromise MXR detoxification systems and as a result these contaminants, also named chemosensitizers, enhancing the toxicity of chemical mixtures (Luckenbach et al., 2004). To date, however, the previous hypothesis has not been fully addressed since the toxicological implications of MXR systems have been restricted to cells and tissues and not to individuals, neither population level responses. Indeed MXR mechanisms in aquatic organisms have been mostly characterized in fish cells, embryos of few cells and mussel gills and hence their toxicological implications are questionable. In this chapter I aimed to characterize the MXR system and its toxicological implications in *D. magna* individuals. This species is the most used toxicological aquatic model today so the results of this chapter can be of a broad interest. The results of

this chapter indicate that reported ABC transporter genes, obtained in an *in silico* study of *D. pulex* genome (Sturm et al., 2009) occur and are differentially transcribed in *D. magna*. Accumulation dye assays indicated that juveniles of *D. magna* have an active MXR system that works similarly to those described in fish cell lines and mussel gills effluxing out from the cells toxic substances and other metabolites. Acute toxicity assay results using binary combinations of MXR inhibitors with MXR toxic substrates also confirmed that inhibition of the MXR system enhanced toxicity. These results, thus, are the first ones relating MXR transporter activity with mortality responses in fully developed organisms of an aquatic species. Previous results have shown that inhibition of MXR systems enhance toxicity of cells, embryos or increased tissue damage in mussels (Faria et al., 2011; Luckenbach and Epel, 2008; Smital et al., 2004) but there is no such study that related MXR system with individual responses. Furthermore, since mortality effects are easily translated to population level effects, the results of this chapter are easily extrapolated to populations and hence may be incorporated in ERA. For example in the past non-polar narcosis mechanisms were incorporated into ERA using QSARs. Similarly dioxin like and estrogenic effects were incorporated into ERA by using Toxic Equivalents relative to model compounds such as BaP or E2 (Van den Berg et al., 1998). To do that, however, it was necessary to test that such mechanisms were broadly distributed across species and that it was possible to model empirically those effects using available toxicological data sets. In this chapter I provide the first evidence that this system exists and is important in maintaining organism fitness in contaminated environments. It may also be of paramount importance to explain synergistic joint toxic effects of mixtures. The synergic effects are environmentally very relevant, because when they are observed it is an indication of a serious threat to ecosystems health. Thus this system can provide a mechanistic explanation of synergism in toxic mixtures. Nevertheless, future work will require testing more substances and probably refinement of our transporter assay system in order to better characterize the system and its activity potential. Will also be important to understand which chemicals can inhibit this system (Luckenbach et al., 2004).

This thesis has been developed with the aim of studying emerging effects of priority and emerging contaminants. We proposed ourselves to develop new methods and conceptual approaches that would allow us to achieve this.

Even though SSRIs, 4-nonylphenol, NM and the defensive mechanism MXR seem very different, they are all emerging substances and/or mechanisms and represent new threats to the environment. Most of the emerging substances are still overlooked or analysed in a very superficial way, and in this thesis I showed that the above mentioned substances have emerging risks that may affect biological levels beyond individuals.

The ultimate aim of ecological risk assessment is to provide sufficient information for decision-making with the purpose of protecting the environment from unwanted effects of chemicals (Breitholtz et al., 2006). Nowadays we face the enormous task of assessing numerous chemicals and complex chemical mixtures while protecting the diversity of ecosystems (Escher and Hermens, 2002). The need for improved safety data from ecological risk assessments is continually increasing; the effects of new and existing chemicals along with the threat of emerging pollutants, such as human and veterinary pharmaceuticals, are all increasing the demand for regulatory testing for ecological effects (Ankley et al., 2007). The work developed in the frame of this thesis can help us to better understand the problems and challenges that we are already facing and help to open new research lines for the future.

7.2. Conclusions

The work developed in the frame of this thesis allow us to draw some conclusions that can be important in the context of better understanding the responses of *Daphnia magna* to stress, and the molecular and physiological modes-of-action through which these changes take effect.

1. Effects of low exposure levels of SSRIs and 4-nonylphenol on life-history traits of *Daphnia magna* varied across developmental stages and food levels. At limiting food levels SSRIs increased the developmental rates and offspring production, while 4-nonylphenol also increased offspring production irrespectively of food levels but at the cost of producing smaller neonates. 4-Nonylphenol effects in *D. magna* are maladaptive, being that these neonates will be smaller than the optimal size.
2. SSRIs had a similar molecular mode of action in *D. magna* than in humans, affecting the serotonin metabolism pathway. It also affected genes involved in neuronal development and synaptic vesicle formation, together with effects in the carbohydrates and Krebs cycle pathways. 4-nonylphenol exposed animals had de-regulation of genes involved in the ecdysone regulatory pathway and also in the carbohydrates metabolism pathways, which is in line with previous findings.
3. Physiological responses showed that SSRIs altered the metabolism of *Daphnia magna*, favouring oxidative catabolism, which is more energy efficient, but at the cost of being more sensitive to anoxia conditions, being this change maladaptive in the context of the vertical diel migration and may have a serious impacts in the food web.
4. The use of new statistical methods (PLS Partial Least Squares) and advanced bioinformatics tools (KEGG Kyoto Encyclopaedia of Genes and Genomes) allow us to link physiological and genomic responses, thus, providing a better insight into the molecular MoA.

5. The presence of NM on the water column lead to the formation of large agglomerates with algae, thus causing depletion on the food availability to *D. magna*, which severely impaired growth, reproduction and population growth rates at limiting food conditions.
6. New experimental approaches are needed to develop tests able to discriminate effects on the food sources and on *Daphnia magna* itself. In this work we present a possible approach, using larger vessels to maximize effects of NM due to aggregation to edible food particles.
7. I provided the first evidence for a functional MXR system in *D. magna* from the gene to the toxicological individual level. *D. magna* has an operative system of trans-membrane transporter proteins similar to those described in human cancer cells. The inhibition of this system enhanced dramatically the toxicity of contaminants that are also known to be substrates of MXR.
8. The assays developed in this study may be used in the future to screen for potential MXR inhibitors and hence to predict more than additive effects of toxic mixtures.

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