THE EFFECT OF ECOTOXICANTS ON THE AQUATIC FOOD WEB AND PREY-PREDATOR RELATIONSHIPS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

SARA BANU AKKAŞ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOLOGY

JULY 2009

Approval of the thesis:

THE EFFECT OF ECOTOXICANTS ON THE AQUATIC FOOD WEB AND PREY-PREDATOR RELATIONSHIPS

submitted by **SARA BANU AKKAŞ** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biology Department**, **Middle East Technical University** by,

Prof. Dr. Canan Özgen
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Zeki Kaya
Head of Department, **Biology** _____

Prof. Dr. Feride Severcan
Supervisor, **Biology** _____

Prof. Dr. Meryem Beklioğlu Yerli
Co-Supervisor, **Biology** _____

Examining Committee Members:

Prof. Dr. İnci Togan
Biology Dept., METU _____

Prof. Dr. Feride Severcan
Biology Dept., METU _____

Prof. Dr. Mümtaz İşcan
Pharmaceutical Toxicology Dept.,
Faculty of Pharmacy, Ankara University _____

Assoc. Prof. Dr. Ayşegül Gözen
Biology Dept., METU _____

Assist. Prof. Dr. Neslihan Toyran Al-Otaibi
Physiology Dept.,
Faculty of Medicine, Baskent University _____

Date: 21.07.2009

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: SARA BANU AKKAŞ

Signature :

ABSTRACT

THE EFFECT OF ECOTOXICANTS ON THE AQUATIC FOOD WEB AND PREY-PREDATOR RELATIONSHIPS

Akkaş, Sara Banu

Ph.D., Department of Biology

Supervisor : Prof. Dr. Feride Severcan

Co-Supervisor : Prof. Dr. Meryem Beklioğlu Yerli

July 2009, 162 pages

There is considerable need for higher-tier aquatic risk assessment and information on toxicant-induced molecular alterations in lower aquatic invertebrates. Thus the current study's priorities were two-fold: a novel approach utilizing higher-tier ecotoxicity bioassay-guided ATR-FTIR (Attenuated Total Reflectance Fourier Transform Infrared) spectroscopy to better understand the impact of the presence of fish predation pressure – mimicked by predator-exuded info-chemicals – on cypermethrin or salinity toxicity to *Daphnia pulex* – key-stone species in lake ecosystems – and ultimately better assess toxicant-induced alterations at both organismal and molecular levels.

This approach indicates that even low concentrations of cypermethrin/salinity had significant molecular and organismal effects on daphnids. Fish kairomone acted as a major factor affecting toxicant severity, interacting antagonistically below a threshold and synergistically above. Moreover, molecular ATR-FTIR spectroscopic results, clearly consistent with organismal responses, showed that both

cypermethrin and salinity lead to decreased contributions of lipid and proteins to the investigated daphnid systems. It is further suggested that the action mechanism of the fish-exuded kairomone occurs via the lipid metabolism of *Daphnia*. Hence, infrared spectroscopic results enabled detection of early molecular alterations, whose effects might not always be observable at the organismal level.

The results of this study clearly indicate that the simplistic nature of standard ecotoxicology tests hinders a precise judgment of threats imposed by chemicals of interest. Furthermore, it has been shown that ATR-FTIR spectroscopy has considerable potential for studies on daphnid responses to varying environmental conditions. Thus, this study presents a starting point for increasing the environmental realism of aquatic risk assessment.

Keywords: Ecotoxicology; Cypermethrin; Salinity; Fish kairomone; Infrared spectroscopy.

ÖZ

ÇEVRESEL KİRLETİCİLERİN SUCUL BESİN AĞI VE AV-AVCI İLİŞKİLERİNE OLAN ETKİLERİ

Akkaş, Sara Banu
Doktora, Biyoloji Bölümü
Tez Yöneticisi : Prof. Dr. Feride Severcan
Ortak Tez Yöneticisi : Prof. Dr. Meryem Beklioğlu Yerli

Temmuz 2009, 162 sayfa

Çok-katmanlı sucul risk değerlendirmeye ve toksik maddelerin alt seviyelerdeki sucul omurgasızlarda neden olduğu moleküler değişimler hakkında bilgiye duyulan ihtiyaç doğrultusunda, bu çalışmanın öncelikli amaçları şöyledir: Yenilikçi bir yaklaşım olan çok-katmanlı ekotoksisite biyotayin-güdümlü ATR-FTIR (Hafifletilmiş Toplam Yansıtma Fourier Dönüşümlü Kızılötesi) spektroskopisi kullanarak, balık sinyali tarafından taklit edilen balık avlanma baskısının, cypermethrin veya tuzluluk toksisitesinin göl ekosistemlerinin kilit türlerinden olan *Daphnia pulex* üzerindeki etkilerini nasıl etkilediğini daha iyi anlamak ve sonuçta toksik maddelerin hem organizma, hem de molekül seviyelerinde sebep olduğu değişimleri daha iyi değerlendirebilmektir.

Bu yaklaşım, düşük konsantrasyonlardaki cypermethrin veya tuzluluğun bile *Daphnia*'nın moleküler ve organizma seviyelerinde istitistiksel olarak anlamlı etkilerinin olduğunu göstermektedir. Balık sinyali toksik maddenin etkilerinin

şiddetini belirleyen temel bir etmen olup, belli bir eşik değerinin altında antagonistik ve üstünde ise sinerjistik bir şekilde etkileşime girmiştir. Ayrıca, organizma seviyesindeki gözlemlerle tutarlı olan moleküler ATR-FTIR spektroskopi sonuçları, hem cypermethrin, hem de tuzluluğun incelenilen *Daphnia* sistemlerindeki lipid ve protein konsantrasyonlarını düşürdüğünü göstermektedir. Ayrıca, balık sinyalinin etki mekanizmasının *Daphnia*'nın lipid metabolizması üzerine olduğu önerilmektedir. Dolayısıyla kızılötesi spektroskopisi, organizma seviyesinde her zaman gözlmlenebilir olamayan erken moleküler değişimlerin belirlenmesini sağlamıştır.

Bu çalışma, basit yapılarından dolayı standart ekotoksikoloji testlerinin, araştırılan kimyasaldan kaynaklanabilecek tehditleri hassasiyetle değerlendiremeyeceğini göstermektedir. Dahası, ATR-FTIR spektroskopisinin *Daphnia*'nın değişken çevre koşullları karşısındaki tepkilerinin araştırıldığı çalışmalarda önemli bir role sahip olabileceğini ortaya koymaktadır. Sonuç olarak bu araştırma, sucul risk değerlendirme çalışmalarının çevresel gerçekçiliğini artırmaya yönelik etkin bir başlangıç noktası oluşturmaktadır.

Anahtar Kelimeler: Ekotoksikoloji; Cypermethrin; Tuzluluk; Balık sinyali; Kızılötesi spektroskopisi.

To my Aunt Mary;
I know you would have been proud.

To my niece, Ada Selen Sonat

ACKNOWLEDGEMENTS

This work was supported by the Environmental, Atmospheric, Earth and Marine Sciences Research Group (ÇAYDAG-104Y308) of the Scientific and Technical Research Council of Turkey (TÜBİTAK). The author was a recipient of a grant within the National Ph.D. Scholarship Programme of TÜBİTAK.

The last eight years I have spent with my advisors have been fruitful thanks to their encouragement, motivation and patience. I would like to thank Prof. Dr. Feride Severcan for her patience and belief in me. My further thanks go to Prof. Dr. Meryem Beklioğlu for her neverending motivation and support. Some ideas developed within this work would not have been possible without the constructive contributions of the members of my thesis examining committee, Prof. Dr. İnci Togan and Prof. Dr. Mümtaz İşcan. I sincerely hope these collaborations continue.

I am who I am because of my family. My most sincere and greatest thanks go to my Mommy. You are always there and that's always been just the right thing! It's always been a pleasure to be able to go home and ask Daddy about school, science and anything else. You can always make me laugh, even during these last months. Abla is always there to make me laugh, listen to me complain, or just be a shoulder. Life is more fun when I can joke about it with Memo. There is no place like home, when we are having girl party with Selen. Aunt Mary, you never forgot my little bugs! And I will never forget you..

I do not remember of a single day passing by in the past years without the smile and joy of Havva. Day and night.. Year after year.. Its been over a decade.. ☺

Distance has never been a problem and will never be: Aysun, you were always there!

Everything would have been unbearable if it had not been for the cheerful company of Gizem. We have overcome so much TOGETHER! Days of experiments, nights of analysis, and times of sorrow..

I do not know from who to start with from my labmates: Biophysics is fun: Nihal, Sevgi, Özlem, Şebnem.. But not without Limnology: İdil, Nihan, Onur, Arda..You have always been there on good days and bad. Nihal and Sevgi; your help during the bad days and neverending patience during days of stress have always brought me the energy to continue. Damla and İdil; I know you are always a lab away for coffee or ice-cream. And everything is so much better from our point of view..

Murat, I will never forget your support on those nights of experiments that lasted for weeks! You just being there made everything easier and your excitement about teaching me things just made it more fun!

Can, I don't know how you do it, but you are always there for all of my questions. No matter how ridiculous!

It is sometimes the old friends who understand you the most: Elif and Ceyhun; no matter what happens in life, friends will be friends!

This time my hugges and kisses go to: Emre, Tonguç, Aydın, Zeynep, Umut, Mehmet, Alper, and Burcu..

Lastly, I am grateful to my friends and colleagues from work for their patience during these last months. It would not have been possible if it were not for the kindness and tolerance of Ahmet Sümer, Elife Özlem Karabacak, and Seda Damla Karaoğlan.

And to all my friends that have made life an even better place..

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER 1

INTRODUCTION

The complexity of pollution limits our knowledge of the ecological effects of pollutants, since there is no clear evidence of the linkages between pollutants and their effects on species and ecosystems. However, it is worth conducting a detailed survey to investigate the effects of pollutants on the world's watercourses because we clearly know that the massive quantities of water in the world cannot absorb pollution indefinitely without devastating effects.

1.1 Environmental Pollution

Pollution, which can simply be defined as the act of contaminating the environment with wastes, has been a worldwide problem for over a century. Holdgate (1979) defined pollution as "The introduction by man into the environment of substances or energy liable to cause hazards to human health, harm to living resources and ecological systems, damage to structure or amenity, or interference with legitimate uses of the environment."

Two general categories of pollution can be distinguished according to the nature of the discharge of pollutants. *Episodic* pollution is the case where the pollution is unpredictable in both time and space. Heavy rainfall, releasing large amounts of acid from soils or causing sewerage systems to overflow; or deliberate discharge of wastes; or accidents may result in pollution episodes. Such cases of exposure to large doses of toxicants over a short duration can lead to *acute toxicity* that is usually lethal. Alternatively, *chronic* pollution is the case where the polluted area

receives discharges continuously or regularly. In such cases of exposure to low doses of toxicants over a long time, organisms are said to experience *chronic toxicity*, which might be lethal or sublethal (Mason, 2002).

Pollution may also be classified according to the source that the pollutants are derived from. Examples for *point-source pollution* are discharges of effluents from sewage treatment plants or wastes from factories. On the other hand, pesticides, fertilizers and acid precipitation may lead to *diffuse (nonpoint-source) pollution* by run-off and land drainage. However, in many ways the latter is a greater problem, simply because most of the attention goes to pollution from point sources and such pollution can often be reduced to acceptable levels with the right legal framework (Mason, 2002).

1.2 Aquatic Ecotoxicology

Ecotoxicology has developed from toxicology, where the targets are human beings. However, the target of ecotoxicology is ecosystems, where individuals do not happen to be the center of attention. It is rather populations or communities, where a multitude of species interact with each other and their surrounding environment (Calow, 1998). Therefore, ecotoxicology investigates structural and functional disturbances induced in the short, medium, and long-term by contamination factors (i.e. physical, chemical, and biological) resulting from direct and/or indirect anthropogenic activities (Boudou and Ribeyre, 1997).

Of the Earth's total resource of water, 97% is in the oceans and thus is too salty to be utilized for drinking, irrigation or industrial purposes. The majority of the remainder is in ice caps, glaciers, or deep underground such that it cannot be utilized. Therefore, the exploitable volume of freshwaters is only 0.003% of the total water resource. Despite the fact that water is replenished by the hydrological cycle, it is likely to become considerably scarcer with the impacts of global warming and population growth. Moreover, the majority of the world's

watercourses are directly or indirectly contaminated through human activities because they receive a diverse array of potential pollutants due to industrial, agricultural, domestic and recreational activities.

Freshwater ecosystems may directly receive toxic chemicals due to, for instance, the direct application of herbicides to control water plants considered to be interfering with human use of watercourses. Additionally, insecticides are frequently applied directly to watercourses to destroy the larvae of mosquitoes, which are the vectors of malaria.

Landfill sites and toxic waste dumps are a major cause of indirect groundwater contamination (Lisk, 1991). It has also been suggested that pesticides can be transported through the air over distances of thousands of kilometers. In other words, aerial inputs of pesticides may also have ecological significance, even for remote and previously pristine areas, such as the poles, where the atmosphere may be the sole carrier of contaminants (Finizio *et al*., 1998; Van Dijk and Guicherit, 1999).

In many situations, agriculture and forestry indirectly add pollutants to freshwaters. Warren and colleagues (2003) remark that for many years pesticides were assumed to be 'ideal' in the sense that they are degraded in the soil since that is where they were often applied. Only recently, during the last decade or so, has the scientific community become aware of the influence of pesticides on aquatic systems. It has been realized that it is inevitable for fractions of applied pesticides to enter adjacent water bodies via routes such as spray drift and runoff (Warren *et al*., 2003; Padovani *et al*., 2004). Hence, now it is accepted that nontarget species living in water catchments of agricultural areas are potentially at risk when they have similar toxicant receptors as the target organisms. For example, it was demonstrated by Matthiessen and co-workers (1995) that the farmland run-off of the insecticide carbofuran into a stream produced the acute sublethal and lethal effects observed in the population of the freshwater shrimp *Gammarus pulex*.

A generalized flow diagram of the impact of pollutants on freshwater ecosystems is presented in Figure 1.2-1. This figure illustrates that comprehending the fate of pollutants requires an understanding of a complex range of factors. The means of exposure to the toxicant together with its formulation affects the physicochemical properties during the initial dispersion. Moreover, the topography, soil type, farming practice (e.g. crop-spraying), vegetation, weather conditions such as the temperature, wind direction and speed, and precipitation following the exposure all influence the distribution, transport and transformation of the pollutants. Furthermore, as can clearly be seen from the figure, while some organisms may be directly affected by the pollutants, others may be indirectly affected by the alterations in the community structure (Mason, 2002; Warren *et al*., 2003).

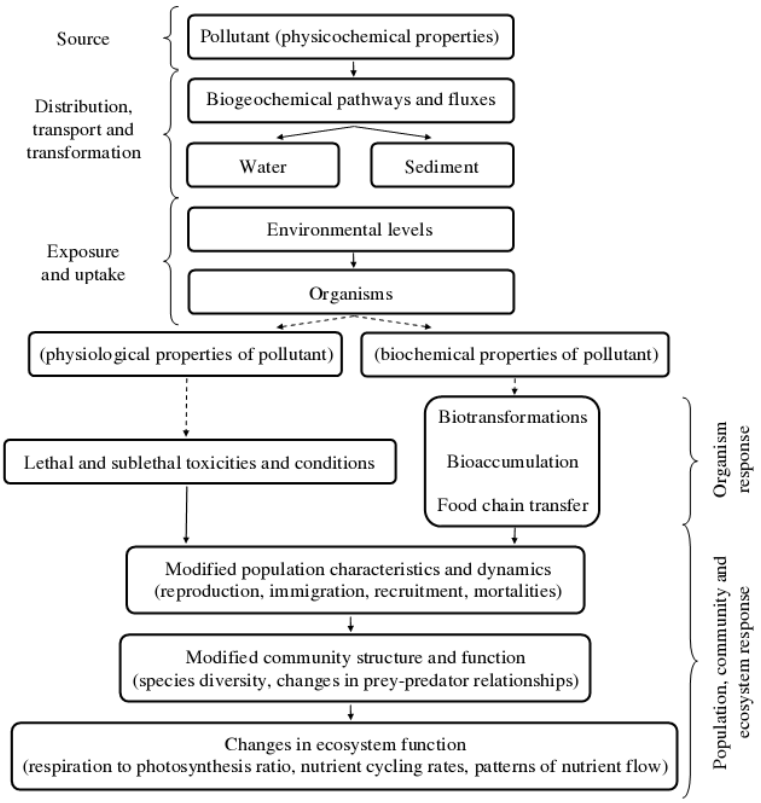

Figure 1.2-1. A basic schematic representation of the impact of toxic pollutants on freshwater organisms and ecosystems (adapted from Connell and Miller, 1984).

Ultimately, the effect of pollutants, which is yet to be explored in detail, is a critical issue during the determination of the status of an aquatic ecosystem in terms of biodiversity and public safety (Moss, 1998).

1.3 Pesticides and Risk Assessment

The United States Environmental Protection Agency generally defined a *pesticide* as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest. The active ingredient is the chemical that performs the pesticidal activity. Pests are regarded as living organisms that are situated where they are not wanted or that cause damage or economic loss or transmit or produce disease. Hence, examples include animals (e.g. insects, mice), unwanted plants (e.g. weeds), fungi, and microorganisms. Consequently, pesticides are generally referred to according to the type of pest they control. Some examples could be algicides, fungicides, herbicides, insecticides, and ovicides.

Risk Assessment – Pesticides are highly toxic compounds introduced intentionally – in contrast to other pollutants – and in large quantities to the environment. Thus, they undergo risk assessment using a tiered approach prior to registration and the procedures for such testing are well-established by several regulatory authorities, such as U.S. EPA, OECD, ASTM, etc. The assessment of the risk that a pesticide poses includes the crucial dimension of assessing the ecological effects of this pesticide on aquatic ecosystems. That is, the impact of pesticides on aquatic ecosystems has always received great interest and concern due to their dispersal from agricultural lands via spray drift and runoff, and consequent persistence in all types of freshwater and marine ecosystems (Mason, 2002).

Current approaches to aquatic toxicology testing are based on internationally approved, standardized laboratory ecotoxicity tests in aquatic test organisms (OECD, 1998; U.S. EPA, 2002a,b; OECD, 2004). The particular requirements for risk assessment are always open to further improvement. In recent decades, there

has been an emphasis on the importance of going beyond such clearly defined lower-tier single-species ecotoxicity tests, as they tend to be less environmentally realistic (Cairns, 1983; Boxall *et al*., 2002). In view of this – together with the difficulties encountered during the interpretation and extrapolation of field and/or mesocosm studies (Maund *et al*., 1997) – new approaches that are intermediary to laboratory-based single-species ecotoxicity tests and field and/or mesocosm studies have been recognized (Boxall *et al*., 2002). In order to move toward more realistic exposure assessment, it has become more common to incorporate several features such as non-continuous pesticide exposure (Maund *et al*., 1998; Cold and Forbes, 2004), population growth phases or developmental life-stages (Pieters and Liess, 2006; Kim *et al*., 2008), multi-species (Taub, 1997; Sánchez and Tarazona, 2002), abiotic factors (Ratushnyak *et al*., 2005; Thomas *et al*., 2008), biotic factors (Gómez *et al*., 1997; Pieters *et al*., 2006; Sakamoto *et al*., 2006) to such "higher-tier" ecotoxicity tests. This could be attributable to their ability to overcome the likely over- or under-estimation resulting from standard single-species toxicity studies (Maund *et al*., 1998; Pieters and Liess, 2006).

Background – Pesticides can be classified according to the common source or production method that they are derived from. Organochlorines, which include compounds such as DDT, endrin, dieldrin, aldrin, chlordane, lindane, endosulfan, and heptachlor, were dominant in the late 1950s and early 1960s. Since they are hydrophobic, lipid soluble and biologically stable, they rapidly pass from water to the tissues of aquatic organisms and biomagnify in the food chain. However, due to their health and environmental effects and their persistence, legal restrictions were placed on the use of many organochlorines in the developed world in the 1980s. Nevertheless, they are still found widely in freshwaters, sediments, fish and animal tissue because of their high hydrophobicity and bioaccumulative properties.

Many modern pesticides have replaced the so-called 'older' organochlorine pesticides. Firstly, organophosphorus pesticides, which usually are not persistent in the environment, replaced some chlorinated pesticides. Subsequently, pyrethroid

pesticides largely replaced the organophosphorus ones. These pesticides were developed as a synthetic version of the naturally occurring pesticide pyrethrin and were modified to have greater stability in the environment. Despite the fact that these pesticides are generally considered not to be persistent, they can be extremely toxic to the aquatic environment because they damage the nervous system.

1.4 Pesticides in Turkey

According to the data from the Food and Agriculture Organization of the United Nations, Turkey consumed about 23,000 tons of pesticides solely in the year 2001. Brazil and Italy consumed 3 fold, while France consumed 4 fold the amount of pesticides that Turkey did in the same year. Figure 1.4-1 shows the increase observed in the agricultural pesticide input in Turkey between the years 1990 and 2001 (World Resource Institute). Moreover, by the year 1998, over 2000 pesticide brands had been licensed in Turkey.

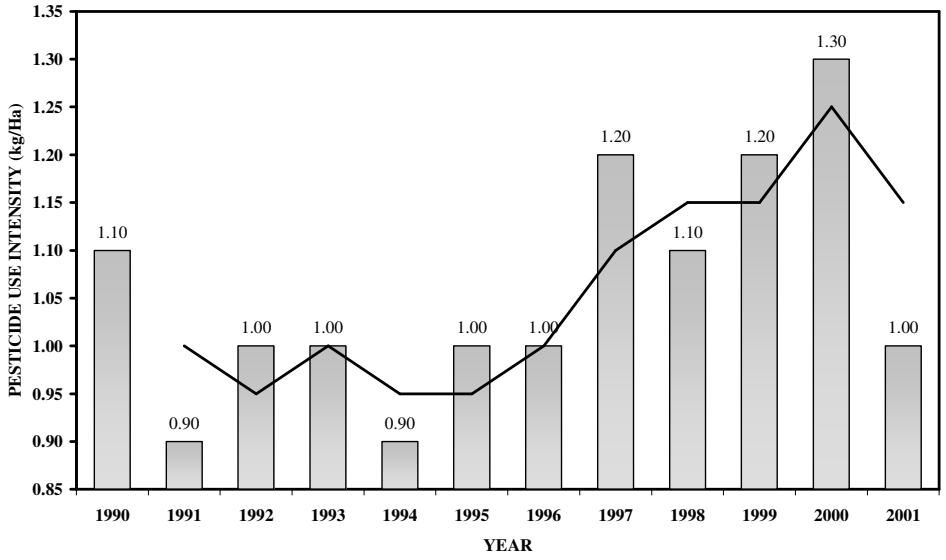

Figure 1.4-1. Trend in the pesticide use intensity given in kg/Ha in Turkey between 1990-2001 (data from World Resource Institute Official Website).

Figure 1.4-2 shows the increase in the number of pesticides that were licensed in Turkey between the years 1993 and 1998 (Dağ *et al*., 2000).

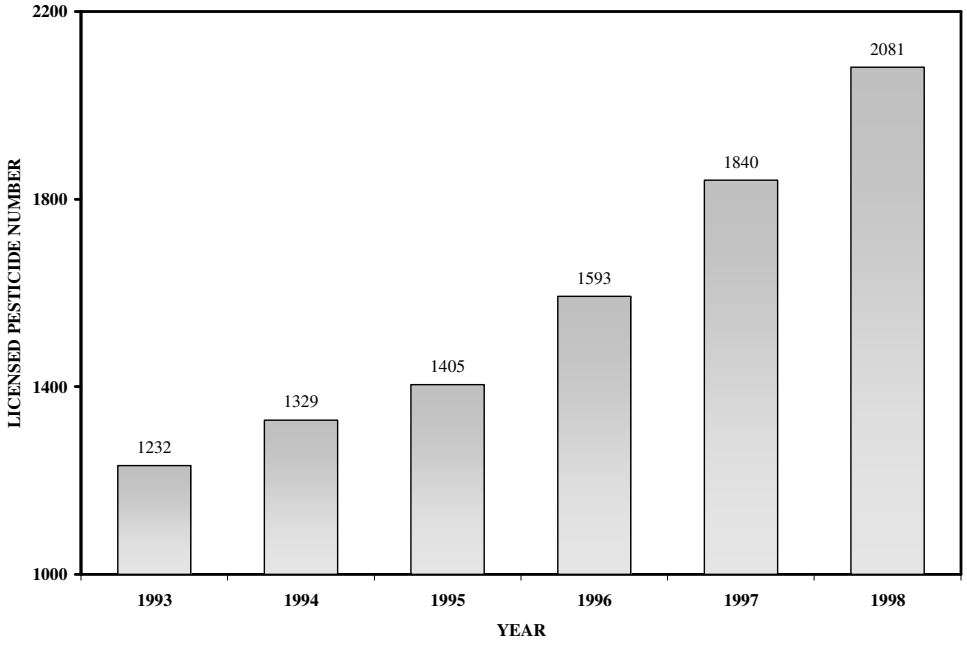

Figure 1.4-2. Number of pesticides licensed in Turkey between 1993-1998 (data obtained from Dağ *et al*., 2000).

As shown in Figure 1.4-3, the major proportion (46% in 1998) of pesticide sales in Turkey is made up of insecticides, of which 40% is organophosphorus insecticides and 21% is synthetic pyrethroids. The major organophosphorus active ingredients include chlorpyrifos, diazinon, dichlorvos, and malathion and parathion methyl, while the most common active ingredients found in the synthetic pyrethroids are cypermethrin, lambda cyhalothrin, tralomethrin, alpha-cypermethrin and zeta-cypermethrin (Dağ *et al*., 2000). Figure 1.4-4 presents the percentages of pesticide types that were used in Turkey between 1980-2004.

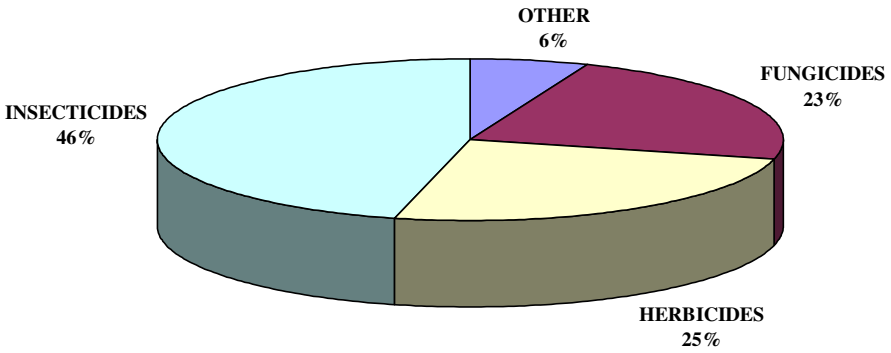

Figure 1.4-3. Proportion of pesticide sales in Turkey (data obtained from Dağ *et al*., 2000).

The majority of pesticide use is for cotton (20.4%) and grain (19.7%) crops. The percentages of pesticides used for different crops are illustrated in Figure 1.4-5. When considered specifically for insecticides, application to cotton fields corresponds to 40%, while 20% is applied to fruit fields (Dağ *et al*., 2000).

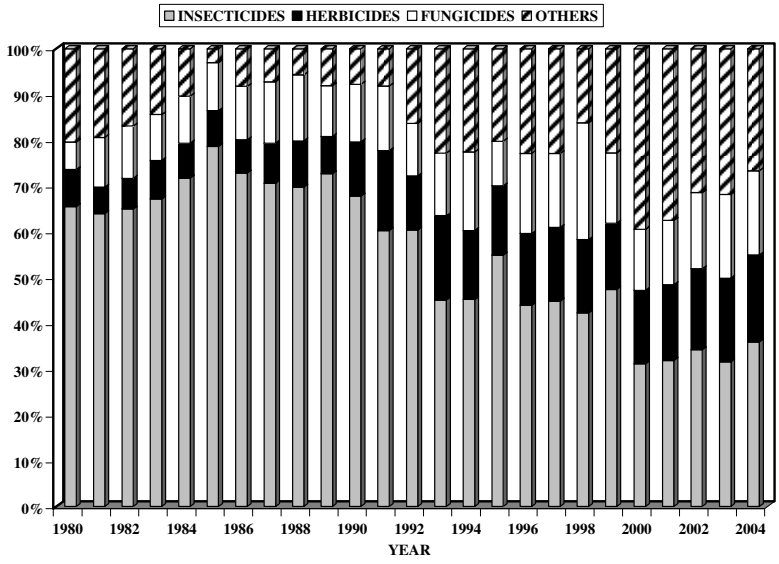

Figure 1.4-4. Percentages of pesticide types used in Turkey between 1980-2004 (data obtained from the Ministry of Agriculture and Rural Affairs).

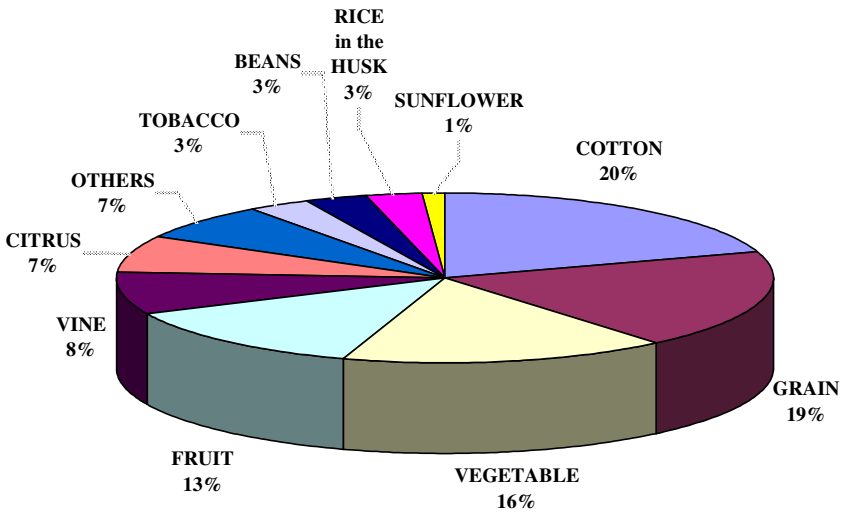

Figure 1.4-5 Percentages of pesticides used for specific crops in Turkey (data obtained from Dağ *et al*., 2000).

Taking into account that the majority of agricultural activities are located near water bodies, it is predestined that pesticides, fertilizers and their derivatives will reach the underground waters. In Turkey, the majority of agricultural activities and thus pesticide usage takes place in the Mediterranean and Aegean regions. According to current data, 40% of the total pesticide consumption in Turkey occurs in the cities of Adana, İçel and Antalya, while 25% occurs in the district of İzmir (Dağ *et al*., 2000). In consequence, the Seyhan and Ceyhan catchment, the Gediz plain, and the Küçük Menderes and Büyük Menderes plains are the regions under considerable threat of aquatic contamination. Moreover, for these regions it is believed that the level of contamination has not been studied in adequate detail and that it is very important to carry out such studies (Burak *et al*., 1997).

1.5 Pesticide Toxicity

Pesticides have become an integral part of the ecosystem, although many of them are extremely toxic, even to non-target species. Hence, there is increasing concern about the effects of pesticides on human, wildlife and ecosystem health. However, it is well known that pesticide effects on different levels of biological organization differ. For instance, at the molecular level, many studies on humans and several animal models have reported pesticide-induced oxidative stress by stimulation of free radical production, induction of lipid peroxidation, and disturbance of the total antioxidant capability of the body (Reviewed in Abdollahi *et al*., 2004).

On the other hand, when ecosystems are considered, it is well known that they encompass large numbers of species forming complex food web interactions at species, population, and community levels. For example, it has been shown that the insecticide diazinon, sprayed at a low-level in an enclosed prairie grassland ecosystem, caused severe reproductive effects on the inhabiting small rodent populations. These changes included a decrease in the incidence of the reproductive condition in males and females, the percentage of pregnant females, and the percentage of females giving birth. The community was also adversely affected because the strong interspecific competition was deteriorated (Sheffield and Lochmiller, 2001). Furthermore, Newton and Wyllie suggest that the recovery of a sparrowhawk population, which was absent for about 20 years in the eastern districts of England, was largely due to the decline of organochlorine pesticide contamination, namely aldrin, dieldrin and DDT and their derivatives (Newton and Wyllie, 1992). On the other hand, a palaeoecological study performed by Stansfield and co-workers suggests that the mechanism that might have caused the switch from submerged plant dominance to phytoplankton dominance in the Norfolk Broads could be the organochlorine pesticide poisoning of the Cladocera community and the subsequent algal domination (Stansfield *et al*., 1989). Thus, these series of examples strictly put forward that pesticides can be toxic to a variety of species ranging from terrestrial to aquatic organisms – such as phytoplankton,

zooplankton, shrimp, fish, mammals, and birds – with not only direct, but also indirect effects (i.e. trophic cascades).

1.6 Pyrethroid Toxicity: Cypermethrin as a Test Compound

Background – Pyrethrins are natural insecticides derived from yellow *Chrysanthemum cinerariifolium* and *Tanacetum cinerariifolium*. However, to attain greater chemical and photo-stability than natural pyrethrins, numerous synthetic derivatives known as pyrethroids have been produced. The commonly used synthetic pyrethroid cypermethrin is a potent and broad spectrum insecticide generally used in order to protect cotton, fruit and vegetable crops (U.S. EPA, 2008). Cypermethrin was chosen as model pesticide in the present study since it is a new-age insecticide commonly used in the Mediterranean region, where a majority of agricultural activities and thus pesticide usage occurs in Turkey (See section 1.4). It is one of the most frequently authorized active ingredients used on arable crop areas in Turkey (Ministry of Agriculture and Rural Affairs, General Directorate of Protection and Control Statistics, 2004). Cypermethrin has the following structure:

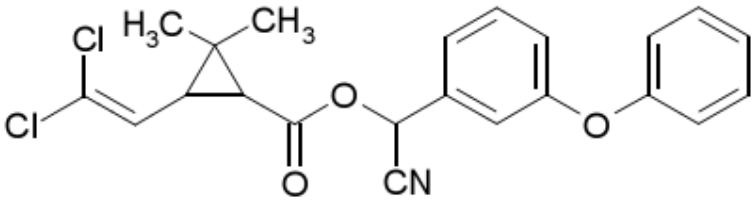

Figure 1.6-1. Structure of cypermethrin

Pyrethroid insecticides, which have been used in agriculture for more than 30 years, account for approximately one-fourth of the worldwide insecticide market (Casida and Quistad, 1998). Total cypermethrin use as an active ingredient (a.i.) per year is approximately 500 tons. It is mainly used in agriculture for mainly cotton and some other crops (pecans, peanuts, broccoli, and corn), as well as livestock treatments, and non-agricultural sites (indoor and outdoor pest control etc.) (U.S. EPA, 2008). Cypermethrin has a relatively high octanol-water partition coefficient (Log K_{ow} = 6.60), and relatively low water solubility (7.6 ppb at 25 °C) (U.S. EPA, 2008). Therefore, it has a high potential for bioaccumulation into organisms.

Pyrethroids contain an acid moiety, a central ester bond, and an alcohol moiety, according to which they are classified as type 1 or type 2. Cypermethrin belongs to the type 2 class. Both types inhibit the central nervous system of insects (Soderlund *et al.*, 2002). Synthetic pyrethroids likely show their toxic action primarily by interfering with the ionic conductance of nerve membranes by blocking some aspect of the synaptic function of the nerve axon. Additionally, they inhibit the integral protein Na+/K+-ATPase in neuronal membranes and consequently disturb the sodium gradient (Kakko *et al.*, 2003). They exert their detrimental effects through delaying closure of the inward sodium channel and so prolonging the sodium current of the nerve membrane (Vijveberg and de Weille, 1985). This results in altered transmission of impulses between nerve cells, increased neurotransmitter release and thus multiple action potentials in nerve cells.

Toxicity – Pyrethroid pesticides are widely used due to their rapid biodegradability, low water solubility and high toxicity to most insect orders and low toxicity to mammals and birds. Unfortunately, these advantages lead to a generous use of pyrethroids. Moreover, pyrethroids are generally formulated with synergists, which increase their potency and ultimately hinder detoxification. These factors have increased the risk of intoxication for non-target terrestrial and aquatic organisms (Smith and Stratton, 1986; Bradbury and Coats, 1989).

Despite synthetic pyrethroids' low toxicity to most non-target animals, such as birds and mammals; they are highly toxic to many non-target aquatic organisms (Smith and Stratton, 1986; Bradbury *et al*., 1987; Day, 1989). This is likely due to taxonomic similarities between the target organisms and non-target aquatic organisms, many of which belong to the phylum arthropoda comprised of insects and crustaceans. Thus, although vertebrate species such as fish are capable to actively biodegrade pyrethroids (Ohkawa *et al*., 1980, Haya, 1989), metabolization of pyrethroids by invertebrate species such as *Daphnia* does not occur (Ohkawa *et al*., 1980). This is likely due to the functioning of liver enzymes – lacking in insects – performing ester hydrolysis and oxidation in mammals and fish (WHO, 1999).

Several studies have investigated the toxicity of cypermethrin to various fish species (Polat *et al*., 2002; Saha and Kaviraj, 2003; David *et al*., 2004). During such toxicity studies with fish, it is emphasized that fish lack the enzyme system that hydrolyzes pyrethroids, which have a high rate of gill absorption due to their lipophilic nature (Demoute, 1989). It has also been shown that fish when exposed to cypermethrin showed erratic movement and frequently visited the water surface, indicating that they depend more on aerial oxygen due to respiratory problems (Saha and Kaviraj, 2003).

Cold and Forbes (2004) investigated the effects of a short pulse exposure on the freshwater shrimp, *Gammarus pulex*, to the pyrethroid insecticide, esfenvalerate. For instance, an exposure of 0.05 μg L^{-1} for 1h led to immediate disruption of reproducing pairs, release of eggs or offspring from the brood pouch and substantial delays in pair formation and subsequent reproduction following transfer to clean water. The results indicated that esfenvalerate has significant effects on survival, pairing behavior, and reproductive output of *G. pulex* even 2 weeks after the pulse exposure. It is important to note that such effects could potentially impact the population dynamics of *G. pulex* in the field.

It has been proposed that cladocerans and copepods are more sensitive to cypermethrin than rotifers (Giddings *et al*., 2001; Mian and Mulla, 1992). A positive relationship between body size and toxicant sensitivity has been proposed such that large-sized cladocerans may be more sensitive to many chemical stressors than smaller ones (Gliwicz and Sieniawaska, 1986; Hanazato, 2001).

It has also been suggested that the early life stages of daphnids and copepods are more vulnerable to cypermethrin toxicity (Wendt-Rasch *et al*., 2003; Willis and Ling, 2004). Moreover, a sublethal exposure of the marine copepod *Acartia clausi* to cypermethrin resulted in an enhancement of egg production and increased activity in the form of erratic swimming (Willis and Ling, 2004).

Another proposal is that the negative effects of cypermethrin can alter the species composition of zooplankton communities. Several studies carried out with marine or freshwater communities put forward that cypermethrin and other pyrethroids reduced both zooplankton density and biodiversity (Crossland, 1982; Farmer *et al*., 1995; Woin, 1998; Friberg-Jensen *et al*., 2003; Medina *et al*., 2004). In these studies, there was a decline in the relative abundance of cladocerans and copepods following pesticide treatment, indicating a direct lethal effect. On the other hand, there was a domination of the community by rotifers, which can be considered as an indirect long-term effect of the pesticide on community structure. Therefore, such severe impacts of pyrethroid insecticides on Cladocera and Copepoda taxa of Crustacea have direct and indirect effects on freshwater and marine communities (Mian and Mulla, 1992; Wendt-Rasch *et al*., 2003; Medina *et al*., 2004). Thus, it is not surprising that several studies have documented an increase in the algal biomass due to a decrease in zooplankton algal grazing in aquatic ecosystems exposed to insecticides (Van Donk *et al*., 1995; Jak, 1997). It is worth emphasizing that such an effect of insecticide exposure on an aquatic ecosystem resembles the alterations caused by eutrophication and thus may add to the adverse effects caused by an increase in nutrient load (Hanazato, 2001; Wendt-Rasch *et al*., 2003). Hence, these results point out that even “non-persistent” pyrethroid insecticides may

produce detrimental effects that result in long-term changes at the ecosystem level of organization (Woin, 1998).

1.7 Global Warming Toxicity: Salinity as a Test Compound

Background – An important aquatic toxicology problem of the present day is not only pesticide pollution, but also the impact of global warming. It is crucial to consider global warming as a major dimension of human activities and conduct research on its direct and indirect effects on the world's watercourses.

The Earth's warming in this century is projected to continue at a rate greater than its global mean. This warming process is increasingly recognized as a climatic force, which is defined as a mechanism that forces the climate to change by altering the global energy balance. Nowadays, global climate change has gained recognition as a potential factor that alters food webs, especially if interacting species respond differently to altered environmental conditions (DeStasio *et al*., 1996; Petchey *et al*., 1999; Straile, 2000; Winder and Schindler, 2004; Emmerson *et al*., 2005).

Of all ecosystems, freshwaters will have the highest proportion of species threatened with extinction due to climate change (Millennium Ecosystem Assessment, 2005), since climate is a major factor responsible for long-term changes of thermal properties and biological processes in freshwater ecosystems, if anthropogenic influences are absent (Carpenter *et al*., 1992). Many regions will suffer a decrease in water resources due to climate change (IPCC, 2007). Mediterranean climate, with dry sub-tropical summers, dominates regions from 32º to 40º north and south of the Equator in the five major regions of the world among which Turkey, being located in the Mediterranean basin between 36-N and 42-N latitudes, lies on a very steep hydrological gradient (IPCC, 2007). Increase in salinity of freshwater ecosystems is an expected result of global warming because of increased evaporation and reduced precipitation. In fact, significantly increased

salinity as a result of decreased lake levels and increased retention time has already been recorded in freshwater lakes located in central Anatolia, Turkey (Beklioglu and Ozen, 2007), supporting the anticipation that salinity will become a chief stress factor in many Turkish lakes.

Toxicity – Given the current climate change projections, climate change might become a major driving force (Sala *et al*., 2000) directly leading to species loss in cases of environmental variables reaching levels that are beyond the ranges individual species can adapt or get acclimated to (Thomas *et al*., 2004). Biodiversity is expected to decline in freshwater habitats in response to climate change at a far greater scale than is true for even the most affected terrestrial ecosystems (Ricciardi and Rasmussen, 1999; Sala *et al*., 2000).

Marine and freshwater ecosystems have experienced many alterations that have been associated with rising water temperatures, and changes in salinity, oxygen levels and circulation patterns (Ahas, 1999; Elliott *et al*., 2000; Gerten and Adrian, 2002; Strecker *et al*., 2004; Winder and Schindler, 2004, IPCC, 2007; Wagner and Benndorf, 2007). Among these abiotic factors (e.g. temperature, salinity etc.) notably influenced by climate change, salinity is an exceptionally influencial factor affecting zooplankton survival and abundance (Williams, 1987; Dodson and Frey, 2001; Wallace and Snell, 2001).

Osmoregulatory activities are very important for freshwater organisms (Baillieul *et al.,* 1996), since they are evolutionarily adapted to conditions of low osmotic pressure (Grzesiuk and Mikulski, 2006). Thus, freshwater zooplankton are very sensitive to increases in salinity – even at salinities as low as 1‰ (Hart *et al.*, 1991) – or to even minor saline intrusions (Schallenberg *et al.,* 2003). Among the responses of daphnids to increased levels of salinity are increased mortality, decreased growth rates, delayed maturity, smaller size at first reproduction, and increased respiration rate (osmotic regulation is associated with metabolic rate) (Hart *et al*., 1991; Arner and Koivisto, 1993; Teschner, 1995; Lignot, *et al*., 2000;

Hall and Burns, 2002; Grzesiuk and Mikulski, 2006; Sarma and Nandini, 2006; Soucek, 2007, Jeppesen, *et al*., 2007).

Moreover, increased salinity levels in freshwater ecosystems affect the dynamics and abundance of rotifers and cladocerans (Akopian *et al*., 2002), decrease zooplankton growth rates (Sarma *et al*., 2002), and decrease total zooplankton, cladoceran, rotifer, and copepod taxonomic richness (Frey, 1993; Green, 1993; Green and Menengestou, 1993; Hammer, 1993; Jeppesen, *et al*., 1994; Garcia, *et al*., 1997; Ramdani, *et al*., 2001).

1.8 Ecotoxicological Studies

An important problem of the present day is the chemical pollution in aquatic ecosystems caused by anthropogenic factors. Simple, rapid and reliable tests are required in order to determine the effects on aquatic organisms of toxicants reaching aquatic ecosystems. The main goals of such toxicity tests are;

- to predict the effects of toxicants on aquatic ecosystems,
- to compare the sensitivity of one or more species to a variety of toxicants or to a specific toxicant under a variety of test conditions,
- to establish rules and regulations required for an appropriate water quality management plan (Baudo, 1987).

Toxicity experiments can be performed in two manners. Acute toxicity experiments are experimental designs in which doses of a toxicant that rapidly (hours) affects the test organism are used and the mobility of the organisms exposed to the toxic substance are observed. On the other hand, chronic toxicity experiments are experimental designs in which the test organisms are exposed to the toxicant for a substantial portion of their lifetime (days) in order to determine the effects of the toxicant on the reproductive output of the organism (OECD, 1984; 1998).

While determining the test organism to be used for such experiments, three criteria are thought to be relevant:

- ecological representativeness in terms of taxonomy and trophic level or niche,
- availability and suitability for laboratory testing,
- existence of adequate background data of their biological and ecological aspects.

When these criteria are taken into consideration, the facts that *Daphnia* (a) are ubiquitous in the holarctic, (b) are a vital part of the aquatic food web – because they are dominant consumers of primary producers and an important source of food for both invertebrate and vertebrate predators, and (c) have been widely used for aquatic toxicity experiments for decades make this genus a highly representative and relevant test organism for aquatic ecosystems (Baudo, 1987).

Even though organisms having different biological characteristics or toxicants having different physico-chemical parameters are used in such studies, it is profoundly important to be able to maintain comparable test conditions. Thus, in the current study the experiments were based on the "OECD Test Guidelines for Testing of Chemicals: *Daphnia* sp., Acute Immobilisation Test and Reproduction Test" as much as possible (OECD, 1984; 1998).

Therefore, ecotoxicological studies typically make use of simple toxicity tests with *D. magna*, *D. pulex*, *Ceriodaphnia dubia,* etc. (Hanazato, 2001; Sarma and Nandini, 2006). However, since the 1980's it has been stressed that higher levels of biological organization have to be included in ecotoxicological research. This is based on the fact that single-species or single-trophic level testing does not accurately predict the 'real world', which is extraordinarily complex due to the presence of countless biotic and abiotic interactions (Cairns, 1983). It has been reported that multi-species or multi-trophic level approaches provide more realistic scenarios during pesticide risk assessment (Barry, 2000; Hanazato, 2001; Wendt-

Rasch *et al*., 2003). Thus, the inclusion of competitive, or predator-prey or abiotic interactions overcome the low environmental realism of simple species-level toxicity testing.

1.9 Zooplankton

In aquatic ecosystems, zooplankton is the main primary consumer group, which feeds on phytoplankton but in turn is preyed upon by planktivorous fish. Herbivorous zooplankton have major effects on the water clarity of freshwater systems because they graze phytoplankton biomass down to low levels. Algal biomass has to be under control for light to be able to penetrate the water column (Brooks and Dodson 1965; Jeppesen *et al*., 2002). Clear water in turn allows the submerged plants to colonize and consequently supports a high biological diversity in the system (Gee *et al*., 1997; Engelhardt and Kadlec, 2001). Therefore, zooplankton undertakes an important role for the sustainability of the clear-water state and the corresponding structure and dynamics of the whole food web of freshwaters.

1.9.1 Significance of *Daphnia*

As mentioned previously, zooplankton happens to be an essential trophic level in aquatic food webs during the assessment of the status of the ecosystem, namely the clear-water state and the turbid-water state. As hypothesized by Brooks and Dodson (1965), large bodied zooplankters are much more efficient at grazing down phytoplankton biomass than their smaller competitors. To be exact, large zooplankton is expected to have a higher capacity to suppress the algal biomass than small zooplankton due to a higher filtration rate and broader food size spectrum. Accordingly, *Daphnia* (Figure 1.9-1), a large-bodied genus belonging to the herbivorous order Cladocera, is exceptionally effective in reducing algal biomass, changing the algal community structure and thus aiding the development of the clear-water state (Burns, 1968; Elser and Goldman, 1990; Sarnelle, 1997;

Matveed *et al*., 2000). Thus, *Daphnia* play a key role in the structure and function of freshwater ecosystems (Moss, 1998). However, it is further suggested that fish forage selectively on larger zooplankton. Hence, *Daphnia* are also more vulnerable to fish predation through their large bodies (Brooks and Dodson, 1965). Thus, predation pressure is a critical biotic factor influencing community structure and characteristics of zooplankton (Lynch, 1980; Zaret, 1980; Kerfoot and Sih, 1987; Macháček, 1991; Hanazato and Dodson, 1992; Stibor, 1992; Mikulski, 2001; Sakwinska, 2002). Consequently, daphnids have evolutionarily developed a wide repertoire of responses (e.g. molecular, physiological, morphological and behavioral) to natural stressors (Machácek, 1991; Reede, 1995; Beklioglu *et al*., 2008).

Figure 1.9-1. *Daphnia pulex*, photographed by Gizem Bezirci at METU Limnology Laboratory in 2007.

1.9.2 Predator Avoidance Strategies in *Daphnia*

For the species of the genus *Daphnia*, one of the best-studied genera in freshwater environments, adaptive phenotypic plasticity is common in order to ensure the future existence of populations. So, they are very plastic in their responses to the chemicals released by their predators. They have been shown to exhibit changes in their morphology, life history traits and behavioral traits as adaptive strategies in order to avoid or counterbalance predation (Tollrian and Dodson, 1999).

Daphnia may exhibit kairomone-induced morphological adaptations, such as changes in size, helmet elongation, neckteeth development, tail spine formation, in order to escape size selective predation (Dodson, 1974; Boersma *et al*., 1998). In other words, *Daphnia* may become "too large" to be easily handled or swallowed by invertebrate predators (Hebert and Grewe, 1985; Dodson, 1988) or "too small" to be easily seen by fish predators that depend on visual communication (Dodson, 1988; Spaak and Boersma, 1997; Boersma *et al*., 1998).

Daphnia have been shown to exhibit kairomone-induced life history changes in traits such as maturation size and time, changes in egg and offspring size, clutch size, and production of sexual eggs (Stibor, 1992; Weider and Pijanowska, 1993; Machacek, 1995; Spaak *et al*., 2000).

It has been documented that *Daphnia* show behavioral adaptations for predator avoidance. Diel Horizontal Migration (DHM) is one such adaptation observed in shallow freshwater bodies and is characterized with a daytime horizontal migration towards the plant bed and nighttime reverse movement into the open water (Visman *et al*., 1994; Lauridsen and Buenk, 1996; Lauridsen and Lodge, 1996). On the other hand, Diel Vertical Migration (DVM) is an adaptive response observed in deep aquatic systems, where zooplankton descend with dawn to the dark, cold, less oxygenated, and nutrient poor hypolimnion and ascend with dusk to the surface

water to graze on algae (Bollens and Frost, 1991; Ringelberg, 1991; Loose, 1993; Spaak and Boersma, 2001).

The above mentioned adaptations either reduce predation pressure or allow high population growth to compensate for high predation pressure (Zaret and Suffern, 1976; Stich and Lampert, 1981; Lampert, 1993; Sakwinska, 1998; 2002; Hanazato, *et al*., 2001) but also include an ecologically relevant cost (Rinke *et al*., 2008).

However, in nature, zooplankton are exposed to toxicants and natural stressors simultaneously. The assessment of multiple-stressor effects on cladocerans is mostly focused on the interactions of contaminants with food concentration or abiotic factors, such as temperature, heavy metals and pesticides (Folt *et al*., 1999; Barata *et al*., 2006; Pieters *et al*., 2006; Pereira and Gonçalves, 2007; Kim *et al*., 2008). However, cladocerans – especially large-bodied daphnids – are naturally under the top-down control of fish/invertebrates. Thus standard ecotoxicity tests may underestimate toxicity (see Hanazato, 2001 for a review).

Few studies have examined the interactive impact of pesticides and predation pressure – more frequently invertebrate pressure (Hanazato and Dodson, 1992; Barry, 2000; Barry and Davies, 2004; Maul *et al*., 2006; Sakamoto *et al*., 2006). For example, it has been pointed out that the *Chaoborus* kairomone and high concentrations of some insecticides induced similar morphological changes, such as development of protuberant structures in *Daphnia*. However, low concentrations of these insecticides were able induce similar alterations in *Daphnia* when they were exposed simultaneously with the kairomone (Hanazato, 1999). It is proposed by Hanazato (1999) that the development of protuberant structures and some other responses to the predator kairomone require *Daphnia* to expend energy. This may reduce the amount of energy that *Daphnia* can allocate to detoxifying the insecticide, therefore increasing the sensitivity of the animal to the toxicant.

Then again, several studies have investigated the effects of salinity and fish predation pressure separately (Hart *et al.*, 1991; Lampert, 1993; Sakwinska, 1998; 2002; Lignot, *et al*,. 2000; Hanazato, *et al*., 2001; Hall and Burns, 2002; Grzesiuk and Mikulski, 2006; Sarma and Nandini, 2006; Soucek, 2007). Nonetheless, the combined impacts of salinity and the fish predation pressure (mimicked by the fish-exuded kairomone) on *Daphnia* remains to be unknown.

As a result, it has been recognized that higher levels of biological organization have to be included in ecotoxicological research. It is realised that single-species or single-trophic level testing does not accurately predict the 'real world', which is extraordinarily complex due to the presence of countless biotic and abiotic interactions (Cairns, 1983). Thus, it is useful to take into account the responses of daphnids to anthropogenic stressors (Day and Kaushik, 1987; Barry, 2000; Christensen *et al*., 2005), simultaneously with the impact of predation pressure.

1.9.3 *Daphnia* as a Test Organism

Daphnia is widely used as a test organism in many fields of biology and was similarly selected as the test organism of the current study. First of all, the genus is ubiquitous, has a pivotal role in many aquatic food webs and its ecology has been well studied. There are few other groups of organisms where the relationship between the individual and its environment can be studied so easily. Moreover, *Daphnia* is easily cultured and has a high reproductive rate. The fact that they perform parthenogenetic reproduction, which excludes genetic variation, makes them well suited for studies of environmentally induced variability (Larsson and Miracle, 1997). Most importantly, many studies have revealed that crustacean zooplankton – including *Daphnia* – are very sensitive to many substances, including insecticides, and that several of their traits are easily quantified (Jak, 1997). Due to their ecological significance (summarized in 1.9.1) and this variety of responses (summarized in 1.9.2), daphnids are among the test species exploited

most extensively in aquatic toxicology as model organisms revealing the risk posed by anthropogenic stressors (Sarma and Nandini, 2006).

1.10 Spectroscopy and its Applications

1.10.1 Electromagnetic Radiation and Spectroscopy

Electromagnetic radiation is considered as two mutually perpendicular electric and magnetic fields, oscillating in single planes at right angles to each other. These fields are in phase and are propagated as a sine wave. E is the direction of the electric field while B is the direction of the magnetic field (Stuart, 1997; Figure 1.10–1).

λ symbolizes wavelength, which is the distance between two peaks. The product of wavelength λ and frequency ν (Hz), which is the number of cycles per second, will give the velocity of propagation of a wave. It follows that:

$c = \lambda\nu$ where c is the speed of light in vacuum ($c = 3.0 \times 10^8$ ms^{-1}).

Electromagnetic radiation covers a wide range of wavelengths and therefore, frequencies. However, in some cases, such as in infrared spectroscopy, the unit wavenumber $\bar{\nu}$ is used for practical reasons. Wavenumber (cm^{-1}) is the number of waves in a length of one centimeter and thus is given by:

$$\bar{\nu} = \frac{1}{\lambda}$$

The energy ($J.mol^{-1}$) of a wave is given by the Bohr equation:

$E = h\nu = hc\,\bar{\nu}$ where h is Planck's constant ($h = 6.626 \times 10^{-34}$ J.s).

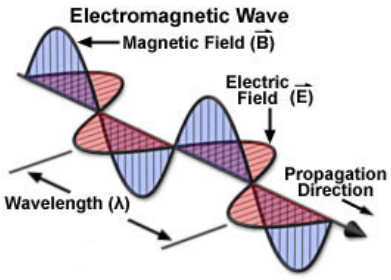

Figure 1.10-1. An electromagnetic wave

The study of the interaction of electromagnetic radiation with matter is called spectroscopy. The plot of the probability of absorption versus the wavelength is called an absorption spectrum. Figure 1.10-2 sums up some of the related energy scales.

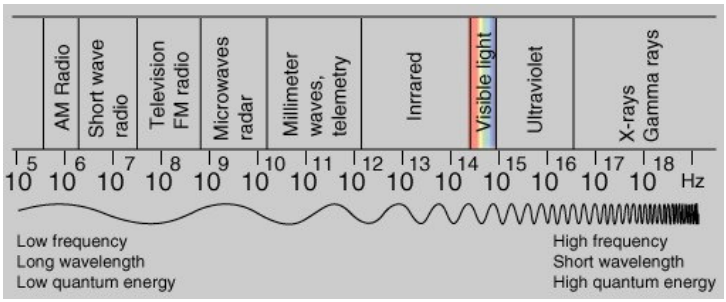

Figure 1.10-2. Electromagnetic radiation and the scales used.

Any molecule has a number of stacks of energy levels, with each stack corresponding to a particular process, such as electronic, vibrational or rotational change. A typical energy-level diagram describing these energy levels is presented in Figure 1.10-3. The thin horizontal lines represent vibrational energy levels. Accordingly, the long arrow exemplifies an electronic transition, while the short arrow typifies a vibrational transition.

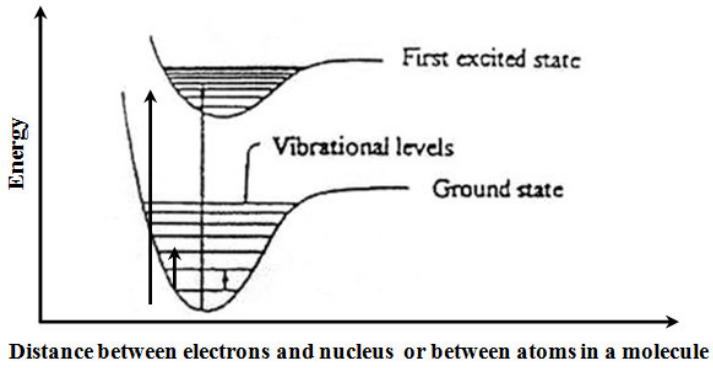

Figure 1.10-3. Typical energy-level diagram illustrating the ground and first excited electronic energy levels and their vibrational levels (Freifelder, 1982).

It is convenient to treat a molecule as if it has several distinct classes of energy:

$$E_{total} = E_{translation} + E_{electron\ spin\ orientation} + E_{nuclear\ spin\ orientation} + E_{rotation} + E_{vibration} + E_{electronic}$$

The contributions of $E_{translation}$, $E_{electron\ spin\ orientation}$ and $E_{nuclear\ spin\ orientation}$ are negligible because the separations between respective energy levels are very small. The separations between the neighboring energy levels corresponding to $E_{rotation}$, $E_{vibration}$ and $E_{electronic}$ are associated with the microwave, infrared and ultraviolet-visible region of the electromagnetic spectrum, respectively (Campbell and Dwek,

1984). The means of study for these energy transitions is standard absorption spectroscopy for electronic transition, infrared and Raman spectroscopy for vibrational and rotational transitions and nuclear magnetic resonance for nuclear spin orientation (Freifelder, 1982).

1.10.2 Infrared Spectroscopy

Infrared spectroscopy has been used in a number of branches of science as a quantitative and qualitative tool. As mentioned earlier, the energy of most vibrational transitions corresponds to the infrared region of the electromagnetic radiation spectrum. Hence, the interaction of infrared radiation with matter is associated with molecular vibrations. An infrared spectrum is usually the plot of absorption as a function of wavenumber ($\bar{\nu}$) and consists of absorption bands with wavenumber maxima expressed in terms of cm^{-1}. The infrared spectrum corresponds to the wavelength range from 10^3 nm to 10^5 nm. This region of electromagnetic radiation is further divided into three regions: the far infrared (400 - 100 cm^{-1}), the mid infrared (4,000 - 400 cm^{-1}) and the near infrared (10,000 - 4,000 cm^{-1}).

The various modes of vibration, which are presented schematically in Figure 1.10-4, involve a change in either bond length or bond angle. The change in bond angle is termed bending vibration, whereas a change in bond length is called stretching vibration, which can either be symmetric or asymmetric depending on the phase of the stretching mode (Stuart, 1997). Consequently, infrared spectra are generated by the characteristic motions of various functional groups (e.g. methyl, carbonyl, amide etc.). The sensitivity of these modes of vibration to any alteration in chemical structure, conformation, and environment presents the value of infrared spectroscopy.

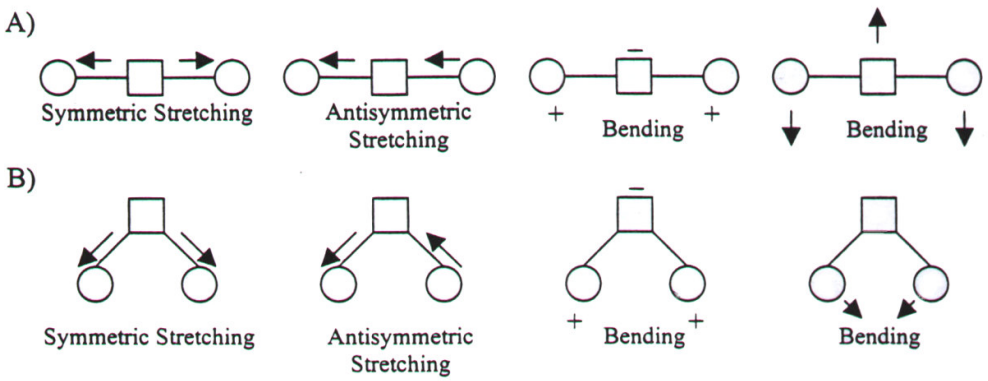

Figure 1.10-4. Schematic representation of some molecular vibrations in linear triatomic molecules (A) and non-linear triatomic molecules (B). (+) and (–) symbols represent atomic displacement out of page plane (Stuart, 1997).

For a vibration to give rise to absorption of infrared radiation, firstly the molecule should have a frequency of vibration similar to electromagnetic wave, and secondly a change in dipole moment should occur.

The reason why infrared spectroscopy is commonly used is that (i) it has been around for a much longer time compared to other tools such as NMR; (ii) sample preparation is very easy; (iii) samples can be prepared in gaseous, liquid, or solid states; (iv) qualitative interpretation is possible; (v) rapid data acquisition is possible, especially with Fourier transform infrared spectroscopy; and (vi) the price is considerably more reasonable, especially compared to NMR spectroscopy (Diem, 1993). Moreover, due to its somewhat different technology, it is used to examine functional groups that are not accessible with ultraviolet and visible light absorption spectrometers (Freifelder, 1982).

1.10.3 Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectroscopy is based on the idea of the interference of radiation between two beams to yield an interferogram, which is a signal produced as a function of the change of pathlength between the two beams. The basic components of an FTIR spectrometer are visualized in Figure 1.10-5. The central apparatus of FTIR instruments is the Michelson interferometer, which causes the incident beam from the source to split into two at a beam splitter. This is followed by the recombination of these two beams at the beam splitter, where they undergo constructive and destructive interference with each other and their transmittance to the sample. This yields an interferogram, which reaches a detector and then an amplifier. The detector observes all frequencies at the same time. The data from the resulting amplified signal is converted to a digital form via an analog-to-digital converter. The signal is then transferred to a computer, where the mathematical method of Fourier transformation of the signal is carried out. Fourier transformation is simply a mathematical means of interconverting distance and frequency in order to sort out the individual frequencies for the final representation of an infrared spectrum (Stuart, 1997).

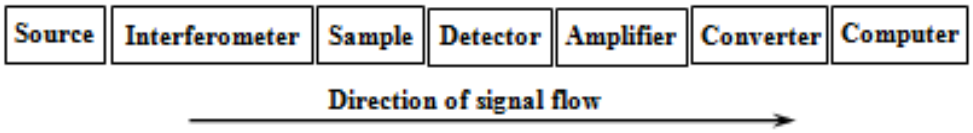

Figure 1.10-5. Schematic instrumentation of the components of an FTIR spectrometer (Stuart, 1997).

The main advantage of FTIR spectrometry lies in its ability to increase signal-to-noise ratio by signal averaging (Stuart, 1997). Moreover, in Fourier transform spectroscopy, it is possible to examine all wavelengths arriving at the detector

simultaneously, compared to being able to sample only one spectral element at a time by the detector (Diem, 1993). Another strength of FTIR spectroscopy is its speed advantage. This makes it possible to obtain spectra on a millisecond scale, which is relatively short compared to other techniques, such as NMR (Haris and Chapman, 1996; Stuart, 1997). Moreover, information about the lipid conformation and protein secondary structure can be obtained simultaneously with a single experiment. What is more attracting is that an idea on the structure and dynamics of molecules can be obtained without the use of any perturbing probe molecules. Samples may be examined in a variety of physical states, such as solids, films, aqueous suspensions and this fact allows the performance of temperature dependent studies because conformational transitions does not present a limitation. Furthermore, small sample quantities are sufficient and *in vivo* studies are possible (Mendelsohn and Mantsch, 1986).

1.10.4 Attenuated Total Reflectance FTIR Spectroscopy

FTIR spectra can be obtained from the transmission or reflection of infrared radiation from the sample. In transmission measurements, the infrared beam passes through the sample. So, all solid, liquid, gas and polymer samples that allow transmission of source light can be investigated. However, in reflectance measurements the infrared beam is reflected from the sample. Reflectance measurements are classified as diffuse reflectance and attenuated total reflectance (ATR) (Stuart, 2004).

ATR is especially useful for opaque solid samples regardless of thickness with a minimum time of sample preparation. Thin films, pastes, powders, suspensions, paper, coatings, and fibers can easily be analyzed with this technique. It is based on the total internal reflection phenomenon. A beam of radiation entering a crystal will undergo total internal reflection when the angle of incidence at the interface between the sample and crystal is greater than the critical angle, where the latter is a function of the refractive indices of the two surfaces.The beam penetrates a

fraction of a wavelength beyond the reflecting surface and when a material that selectively absorbs radiation is in close contact with the reflecting surface, the beam loses energy at the point at which the sample absorbs (Figure 1.10-6). The resultant attenuated radiation is measured and plotted as a function of wavelength by the spectrometer and gives rise to the absorption spectral characteristics of the sample (Stuart, 2004; Günzler and Gremlich, 2002).

The focusing crystals used in ATR cells are made from materials that have low solubility in water and are of a very high refractive index. Such materials include zinc selenide (ZnSe), germanium (Ge) and thallium-iodide (KRS-5) (Stuart, 2004).

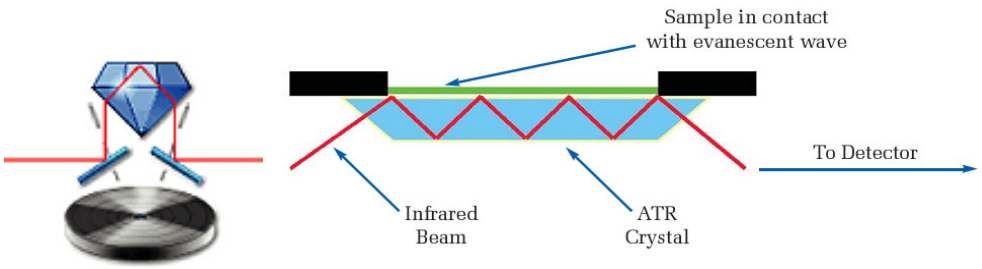

Figure 1.10-6. Schematic representation of a typical attenuated total reflectance cell.

1.10.5 Advantages and Applications of FTIR and ATR FTIR Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy is a rapid, reagentless, direct, non-destructive physicochemical technique based on the detection of molecular vibrations. It enables the quantitative and qualitative identification of the molecular constituents of a variety of biological samples. FTIR spectroscopy has great potential since it can be employed during metabolic fingerprinting, pesticide screening, taxonomic identification, phycological research, atmospheric chemistry

etc. (Cakmak *et al*., 2003; 2006; Columé *et al*., 2004, Luz, 2006; Dean *et al*., 2007; Garip *et al*., 2007; Gorgulu *et al*., 2007; Patel *et al*., 2008).

One of the most useful aspects of FTIR spectroscopy is the fact that different classes of chemical compounds contain structural units that absorb infrared radiation at similar frequencies and intensities. Thus, such characteristic spectral features can simply be assigned to the particular groups or bonds in the corresponding molecules. This approach in vibrational spectroscopy is referred to as group frequencies (Diem, 1993; McKelvy *et al*., 1996). Hence; spectra can be examined in several groups depending on the type group frequency.

The structural and functional information available from an FTIR spectrum are reflected from the form of band shapes, peak intensities, bandwidths, frequency shifts and integrated intensity of the vibrational bands (Cakmak *et al*., 2006; Simsek-Ozek *et al*., 2009). Some more added advantages of FTIR spectroscopy are as follows:

- ✓ Spectra of almost any biological material can be obtained in a wide variety of environments. Thus, spectra can be obtained from biological samples both in solution and in the solid state.
- ✓ It provides a precise measurement method, which requires no external calibration.
- ✓ Small sample quantities as low as few micrograms are sufficient to analyse and *in vivo* studies are possible.

FTIR spectroscopic imaging in the ATR mode is a powerful tool for studying biological samples (Kazarian and Chan, 2006). The most important advantages of ATR-FTIR spectroscopy are as follows:

- ✓ A pure spectrum of any kind of sample – including liquids and solids – is obtained easily in a short time as compared to transmission spectroscopy.
- ✓ ATR mode enables the rapid collection of spectra because it requires minimal or no sample preparation prior to spectral acquisition. This is due to the fact

that the penetration depth of infrared light in the sample for ATR measurements is independent of sample thickness (Kazarian and Chan, 2006).

- ✓ Other techniques, such as infrared transmission, often require the sample to be heated, pressed or ground in order to collect the spectrum. These processing steps for transmission analysis take time and can cause structural changes to the sample. In addition, samples that must be diluted for transmission analysis are usually mixed with salts which may have spectral features of their own.
- ✓ Most samples can be run in their natural state by simply placing a sample in contact with a special, high-index of refraction, crystal (Luz, 2006).
- ✓ ATR-FTIR spectra have better signal-to-noise ratio and increased sensitivity.
- ✓ ATR-FTIR spectra are similar to absorption spectra, but without significant spectral distortion.
- ✓ Quantitative assessment of the different molecular groups can be determined by means of the area under the respective bands.
- ✓ Compared to transmission experiments, it avoids the handling problems that are caused by the required short pathlength.
- ✓ ATR-FTIR spectra are useful in protein structure analysis.

In the current study, ATR-FTIR spectroscopy was applied as a tool for ecotoxicological studies of a freshwater system. Similarlay, a detailed literature survey presents that FTIR spectroscopy is a powerful tool coupled with experimental ease in the course of molecular characterization – both structurally and functionally – of biomolecules such as lipids, proteins, nucleic acids, and carbohydrates for scientific enquiries with ecological perspectives (Giordano *et al*., 2001; Cakmak *et al*., 2003, 2006; Hirschmugl *et al*., 2006; Mecozzi *et al*., 2007).

When organisms are exposed to toxicants in the environment, they certainly experience metabolic costs. Hence, it is crucial to lighten the impact of pesticides at the molecular level, as each preceding level of biological organization is responsible for its sequel. In other words, a population response is actually a molecular response followed by cellular, tissue, organ, and finally organismal

responses, in the given order (Buckler and Tillitt, 1996). Therefore, the application of FTIR spectroscopy in this study is promising in the sense that the interaction of toxic agents with biological systems causes molecular modifications, which can be studied via FTIR spectroscopy (Cakmak *et al*., 2003; Cakmak *et al*., 2006; Mecozzi *et al*., 2007).

1.11 Scope and Aim of the Study

Natural ecosystems are more complex and variable than mimicked in laboratory standardized systems. Thus, there is a major need to go beyond lower-tier single-species toxicity testing in order to assess toxicant risk more accurately, because these tests can seldom be used to assess toxicity in systems with higher levels of biological organization. A more holistic view of the potential damage of a toxicant requires the incorporation of biotic interactions into standard ecotoxicity tests. Consequently, a major aim of the current thesis was to determine the joint effects of toxic stress and fish predation pressure on an important ecotoxicological test species, *Daphnia pulex*, for an improved understanding of daphnid ecology.

Within this context, it is also essential to utilize multidisciplinary approaches as the complementarity between different disciplines will make ecotoxicologists better understand the mechanisms and risks involved in contamination. In accordance, studies aiming to correlate toxicant-induced disturbances at supra-organismal (i.e. survival, growth, reproduction and behavior) level with those at sub-organismal (i.e. cellular and molecular) level are crucial. The majority of such studies focus on fish as test organisms (Adams *et al*., 2000), with recent attention to invertebrate test species – as well as daphnids (De Coen and Janssen, 1998; Christensen *et al*., 2005; Vandenbrouck *et al*., 2009). Such studies are based on the fact that metabolic adjustments are stimulated in order to compensate for the disturbances resulting from toxic stress. Accordingly, in the present thesis ATR-FTIR spectroscopy was selected as a tool proven capable of probing metabolic alterations and attaining

compositional and structural information about the biomolecules constituting the test organisms.

In the currenty study, the following higher-tier approaches were employed: (1) Incorporation of fish-exuded kairomone into ecotoxicity tests using *Daphnia* in order to reveal whether the presence of predator-prey interactions alters toxicity; (2) Detection of toxicant-induced alterations at both organismal and molecular levels, where the former depends on the latter. It is hypothesized that these two novel approaches will provide new insights to environmental risk assessment.

In short, the general objective of this thesis was to estimate toxicant risk more realistically by going beyond single-trophic level toxicity testing via parallel toxicity testing incorporating more than one level of organization with the inclusion of predator-prey interactions and detection of effects at both organismal and molecular levels. The specific objective was to observe the survival, reproductive and molecular responses of *Daphnia pulex* exposed to cypermethrin and salinity – as antropogenic stressors – in the presence of the fish-exuded kairomone – a natural stressor mimicking predation pressure – by means of ecotoxicity bioassays complemented with ATR-FTIR spectroscopy.

1.12 Novel Aspects of this Study

There are three noveltiesof the current thesis that may be of major contribution to the related scientific liteture:

- ✓ Incorporation of higher levels of biological organization into single species tests, which specifically may provide a better understanding of the joint effects of toxic stress and predation pressure on *D. pulex*,
- ✓ Integration of survival and life-history trait data with spectroscopic data, which may reveal the action mechanisms of toxicants and in particular enable the detection of early molecular alterations, whose effects may not always be observable at the organsimal level,

- ✓ Implementation of spectroscopic data into ecology, which may actually be a pioneer interdisciplinary approach to ecological phenomenon

CHAPTER 2

MATERIALS AND METHODS

This research can be divided into two major parts: cypermethrin toxicity and salinity toxicity. Moreover, these two major issues were investigated utilizing two approaches in a joint manner: standard ecotoxicity testing and Fourier Transform infrared spectroscopic analysis. The methodology employed throughout this thesis will be presented in the following section.

2.1 Test Organisms

Test animals are *Daphnia pulex* (De Geer) originating from Lake Eymir (Ankara, Turkey), where they coexisted with fish and invertebrate predators. Test organisms were originally collected a decade ago, after which they were maintained in a climate room with a temperature of 22±1 °C and a photoperiod of 16h light : 8h dark (Beklioglu *et al*., 2006). The cultures were fed regularly with fresh chemostat-grown *Scenedesmus obliquus* (Culture Collection of Algae (SAG) University of Göttingen, Germany), which was cultured in the proteose-peptone medium.

The *D. pulex* cultures were acclimated to the COMBO culture medium (Kilham *et al*., 1998) in the laboratory under constant conditions with regular renewal of the COMBO medium (pH 7.50±0.05). The *D. pulex* population grown in the COMBO culture medium was followed for three generations in order to remove maternal effect (Vijverberg, 1989; Doksæter ve Vijverberg, 2001) and to allow for the acclimation of the population to the COMBO culture medium. The generation follow-up design is presented schematically in Figure 2.1**–1**.

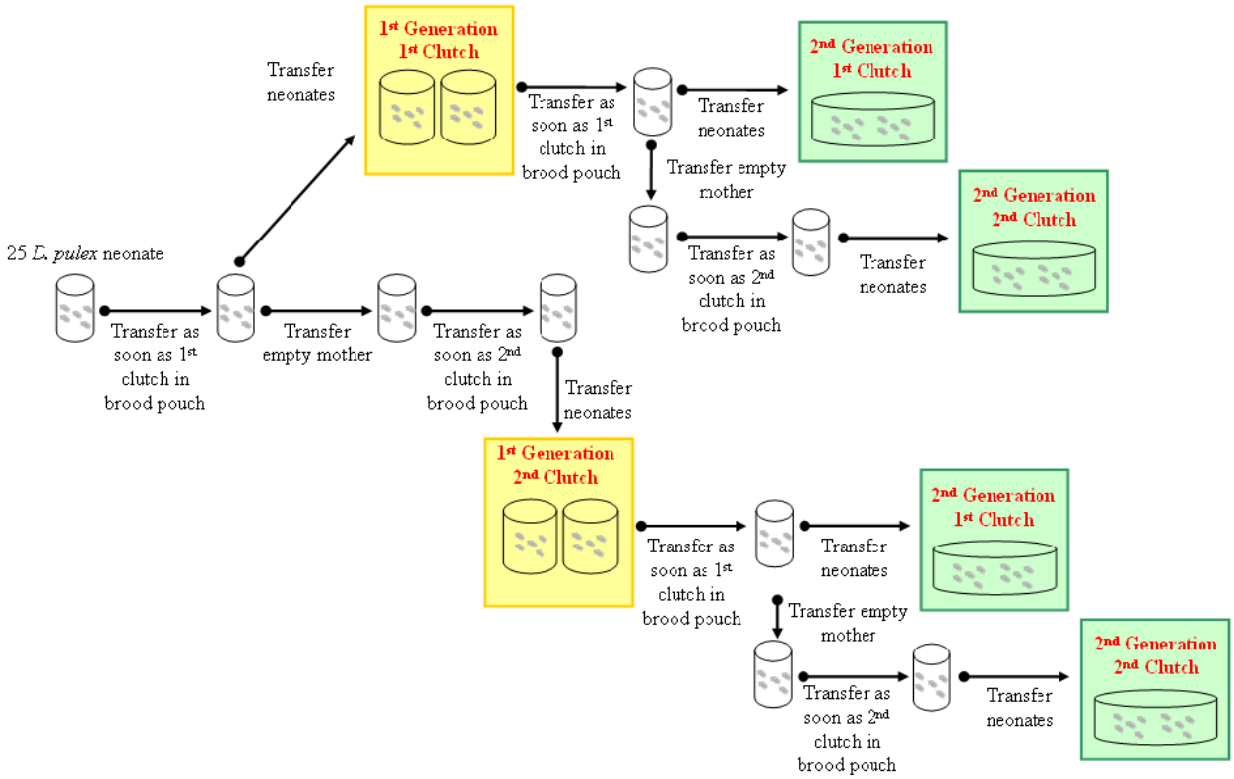

Figure 2.1–1. Generation follow-up design for *D. pulex* in COMBO culture medium.

During this phase, special care was taken such that there are never more than ~50 individuals in ~1L COMBO culture medium, to prevent the occurrence of the crowding effect. Moreover, the cultures were fed regularly with *S. obliquus* at a concentration well above the incipient limiting level of carbon for *Daphnia* (Lampert, 1987). This acclimation phase was carried out in order to allow the daphnids to physiologically adjust to a defined culture medium and thus ensure consistency by eliminating maternal effect and the effects of the toxicants in the lake water (Hall and Burns, 2002; Kashian and Dodson, 2002). After this acclimation phase, newly born neonates of at least the 3rd generation were separated from the stock cultures prior to the experiments and randomly assigned to treatments (Vijverberg, 1989).

2.2 Preparation of Treatments

The *Daphnia pulex* species to be handled during the course of this research were transferred to a medium called COMBO culture medium that has been defined by Kilham and co-workers (1998). It is widely accepted in the literature that this culture medium is adequate to support the growth of zooplankton. Moreover, the utilization of a defined freshwater medium helps increase the compatibility between experiments and allows for the avoidance of any chemical or biological toxicant possibly present in lake water to interfere with the experiment. The preparation of the COMBO culture medium is presented schematically in Figure 2.2–1. As seen from the figure, prior to use the medium is filtered through a 0.22μm pore-sized membrane filter, adjusted to a 7.0<pH<8.0, and then stored at 4°C if necessary (Kilham *et al*., 1998).

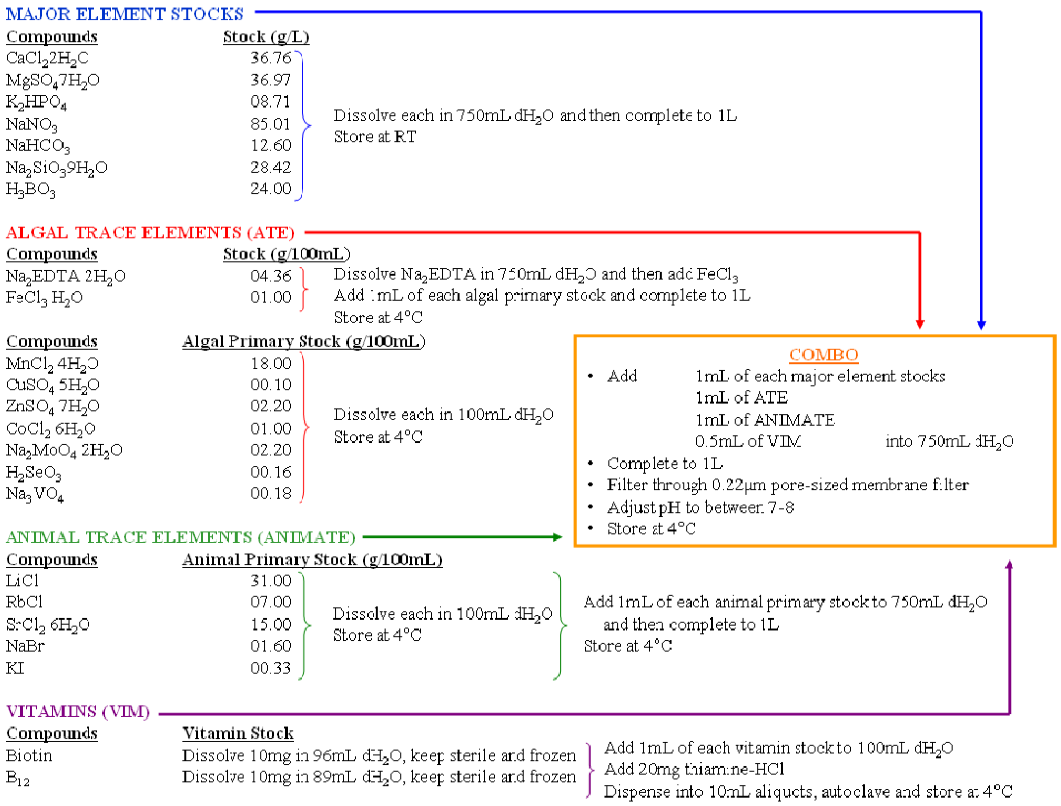

Figure 2.2–1. Preparation of COMBO culture medium (Kilham *et al.*, 1998).

The COMBO medium was prepared fresh with pure water (deionized and distilled) and high-grade chemicals, and kept in airtight bottles under the conditions of the climate room. It was filter sterilized prior to the experiments/renewals by filtering through 0.22 µm pore-sized cellulose-nitrate membrane filters (Millipore Corporation, Bedford MA, USA). Its pH was set to 7.50±0.05. This COMBO medium was then termed the control medium and used in the treatments absent of the fish-exuded kairomone (F-).

For the fish-conditioned medium, the fish used were collected from a nearby lake, maintained in the climate room and fed regularly with synthetic food in order to prevent the warning of daphnids by alarm signals released by injured conspecifics (Pijanowska, 1997). For the preparation of fish-conditioned medium, 10 L of control medium was enriched overnight with 2 medium-sized bleaks (Cyprinidae, *Alburnus escherichii* Steindachner, 1897, body length: 10-15 cm). This is an established protocol determined from the literature (Loose *et al*., 1993; Beklioglu *et al*., 2006), despite the fact that the precise nature of fish-exuded kairomone still has not been fully characterized. Hence, an analytical method to measure both the presence and quantity of the kairomone is yet to be defined (von Elert and Stibor, 2006; Beklioglu *et al*., 2006). This fish-incubated medium was first filtered through 0.45 µm pore-sized membrane filters; secondly filter sterilized with 0.22 µm pore-sized membrane filters and then finally the pH was adjusted to 7.50±0.05. This medium was then used in treatments characterizing the presence of the fish-exuded kairomone (F+).

For the cypermethrin (CM) treatments, a mixture of isomers of the chemical (Fluka, PESTANAL, Seelze, Germany) was tested at concentrations ranging from 0.5 ng/L to 5 µg/L. Cypermethrin was dissolved in high-grade acetone (Riedel-de Haën, Seelze, Germany) and completed with COMBO test medium to relevant stock concentrations. Acetone was equalized for all test treatments, such that all final test media, including the solvent control treatment, contained identical final acetone concentrations that were clearly below the recommended limit (i.e. ≤ 0.1

ml/L = 100 µl/L) reported by the OECD guidelines not to be toxic and not increase the water solubility of the toxic substance (OECD, 1998). Cypermethrin stock solutions were made fresh daily and the desired experimental concentrations were achieved through a series of dilutions and thus were estimated and not verified (Kashian and Dodson, 2002).

Salinity refers to the concentration of dissolved salts in water. For the preparation of salinity treatments, NaCl was chosen as a salinity source. Ionic activities of Na^+ and Cl^- are natural factors affecting the osmotic balance for freshwater organisms, such as crustaceans. In other words, alteration of the ionic composition and loss of osmotic regulation mechanism are mostly related with changes in the ionic compositions of Na^+ and Cl^- (Lignot, 2000; Heugens, 2003). Moreover, responses to NaCl salinity are not different from other salts, as revealed by Cowgill and Milazzo (1990) and Soucek (2007), who observed that sulfate and chloride salts of sodium have similar effects on reproduction.

2.3 Cypermethrin Toxicity Tests

Most products of cypermethrin are mixtures of its isomers. Toxicity of cypermethrin depends on the composition of mixture as well as on the ratio of *cis* and *trans* isomers in the mixture. *Cis* cypermethrin has been found more toxic than its *trans* form to mammals; but for trout, the *cis* and *trans* isomers have been found equitoxic (Bradbury and Coats, 1989). Thus, in this study a cypermethrin product consisting of a mixture of its isomers was used.

The solubility of a pesticide is an important factor affecting its toxicity. Toxicity of pyrethroid pesticides to aquatic organisms depends largely upon their solubility in water. All synthetic pyrethroids are not equally soluble in water. For instance, acetone-solubilized deltamethrin was found to be more toxic than its aqueous solution to freshwater catfish *Clarias geriepinus* (Datta and Kaviraj 2003), while aqueous cypermethrin was found to be more lethal than its acetone-solubilized

form to another freshwater catfish species *Heteropneustes fossilis* (Saha and Kaviraj, 2003). However, since majority of the studies dealing with cypermethrin toxicity to zooplankton species dissolved cypermethrin in acetone, in this study it was preferred to solubilize cypermethrin in pure acetone (Friberg-Jensen *et al*., 2003; Wendt-Rasch *et al*., 2003).

The toxicity tests were carried out with *D. pulex* neonates (24-48 hrs old), which were previously acclimated to the COMBO culture medium for at least three generations. The experiments were performed in the temperature (22±1 °C) and light-controlled (16h light : 8h dark) climate room. The toxicity tests carried out in order to evaluate the combined impact of cypermethrin and fish predation pressure were performed in moderate compliance with the standard protocols reported by the Organisation for Economic Co-operation and Development (OECD, 1998).

In the current study, the concentration-dependent effect of cypermethrin was investigated with three experiments designed with decreasing cypermethrin concentrations. These three levels of cypermethrin were denoted as high, intermediate, and low concentrations. The high concentrations were tested with an acute toxicity test and the other concentrations were tested with two chronic toxicity tests. All sampled individuals were stored at -4 °C for later processing with FTIR spectroscopy.

2.3.1 High-Concentration Acute Toxicity Test

The acute concentrations of cypermethrin were selected in accordance with the range of EC50/LC50 values determined from the literature (Stephenson, 1982; Day, 1989; EPA ECOTOX Database). Cypermethrin test solutions were prepared with COMBO test media standing for both the absence and presence of the fish-exuded kairomone at CM concentrations of 0.0, 1.0, and 5.0 μg/L, together with a solvent control solution. The experiment contained four replicates per treatment. Each replicate had 5 *D. pulex* neonates in 25 ml of the relevant test solution. The

mortality – identified by complete immobilisation – of the exposed individuals were examined at 2 hour intervals for 48 hours, during which no food was provided and no test solution renewal was carried out.

2.3.2 Intermediate-Concentration Acute Toxicity Test

The intermediate concentrations of cypermethrin were selected in accordance with the results of the high-concentration acute toxicity test of the current study. The cypermethrin test solutions were prepared with COMBO test media standing for both the absence and presence of the fish-exuded kairomone at CM concentrations of 0.0, 0.04, 0.10, 0.30, 0.90, 1.80, and 3.60 µg/L together with a solvent control solution. These concentrations (0.04-3.60 µg/L) are in accordance with the concentration range of the above mentioned acute toxicity test (1.0-5.0 µg/L). The experiment contained seven replicates per treatment. Each replicate had 1 *D. pulex* neonate in 40 ml of the relevant test solution. The survival of the exposed individuals was examined daily for 15 days, while the test solutions were renewed every 3rd day in order to refresh renew the toxicant (Kashian and Dodson, 2002). Throughout the experiment, the individuals were fed daily with *S. obliquus* at a concentration of 1.00 mg C/L, which is well above the incipient limiting level of carbon for *Daphnia* (Lampert, 1987).

2.3.3 Low-Concentration Acute Toxicity Test

In the re-registration eligibility decision for cypermetrin, it is pointed out that the lowest reported toxicity value of CM for freshwater invertebrates was an LC_{50} of 0.0036 µg a.i./L for waterfleas (a.i. stands for active ingredient) (U.S. EPA, 2008). In this report it is further detailed that information on the chronic toxicity of CM to freshwater invertebrates is not adequate. For this reason, a surrogate value for the No Observed Adverse Effect Concentration (NOAEC) was derived from the acute and chronic CM exposure data determined for estuarine/marine invertebrates. This surrogate NOAEC value was 0.00059 µg a.i./L (U.S. EPA, 2008). The test

concentrations of the current low-concentration chronic toxicity test were selected accordingly.

Cypermethrin test solutions were prepared with COMBO test media standing for the absence and the presence of the fish-exuded kairomone at CM concentrations of 0.0, 0.5, 1.5, 3.0, 5.0, 7.5, 11.5 and 15.0 ng/L. The range of the treatment concentrations of this experiment (0.5-15.0 ng/L) is slightly lower than the lowest concentration of the above mentioned chronic toxicity test (0.04 μg/L=40.0 ng/L). A solvent control solution was not necessary because the final solvent concentration was too low to be measured within accuracy limits and was significantly less than the final solvent concentrations of the previous experiments, where no significant effect of the solvent treatment was observed compared to the control treatment.

The experiment contained ten replicates per treatment. Each replicate had 1 *D. pulex* neonate in 40 ml of the relevant test solution. The exposed individuals were examined daily for survival for 20 days. The test solutions were renewed every 3rd day, while the individuals were photographed (Leica M Stereo Microscope –MZ16 with attached DFC280 Camera) every other day. Photographing enabled the observation of the adaptive life history traits by precisely recording number of eggs per female (clutch size) and thus determining age at first reproduction (time required for a neonate to become an egg-bearing adult). Throughout the experiment, the individuals were fed daily with *S. obliquus* at a concentration of 1.00 mg C/L.

2.4 Salinity Toxicity Tests

Different concentrations of NaCl, ranging from 0.05-10.00 g/L, were prepared with the COMBO culture medium that was freshly prepared (for details refer to 2.2).

In the current study, three experiments were designed in order to investigate the concentration-dependent effect of salinity. Two acute toxicity tests and one chronic toxicity test were conducted to observe the impacts of both salinity and fish-exuded kairomone on daphnid survival. The former acute toxicity experiment was the basis of the latter acute toxicity test and chronic toxicity experiment. Moreover, the impact of salinity on the life history traits of *Daphnia* were also investigated with another chronic toxicity experiment, which was corroborated with an acclimation phase of daphnids to respective salinity concentrations to mimic salinity adaptation in a real ecosystem.

The toxicity tests carried out were performed in moderate compliance to the standard protocols reported by the Organisation for Economic Co-operation and Development (OECD, 1998 and 2004). All the experiments were performed in a temperature (22±1 °C) and light (16h light: 8h dark) controlled climate room. 24-48 hours old *D. pulex* individuals, which were already acclimated to the COMBO culture medium for at least two generations, were used in all of the toxicity experiments. All sampled individuals were stored at -4 °C for later processing with FTIR spectroscopy.

2.4.1 Acute Toxicity Experiments

Acute toxicity tests are designed to provide concentration-response information, expressed as the concentration that is lethal to 50% of the test organisms (LC50) within the prescribed period of time (24-96 h). Five *D. pulex* individuals were placed into 25 ml of the relevant test solution prepared with COMBO culture media standing for both the absence and presence of the fish-exuded kairomone. Each

treatment had four replicates. The first acute toxicity experiment had 0.00, 2.00, 4.00, 6.00, 8.00 and 10.00 g/L NaCl treatments in the absence (F-) and presence (F+) of the fish-exuded kairomone. The latter acute toxicity experiment was composed of 0.00, 0.20, 0.60, 1.20, 2.25, 3.50, 5.00 and 6.50 g/L NaCl treatments in the absence (F-) and presence (F+) of the fish-exuded kairomone. The mortality of the exposed individuals was identified by complete immobilization and was examined periodically every 2 hours for 48 hours. During the experiments, neither the test solutions were renewed and nor were the animals fed.

2.4.2 Chronic Toxicity Experiments

Chronic toxicity tests are designed to provide concentration-response information during the exposure to a toxicant over two to three weeks and the data attained is used to determine the impacts of the toxicant on the survival and reproduction of the test organism (OECD, 1998 and 2004). One test animal was placed into 40 ml of the relevant test solution prepared with COMBO culture media standing for both the absence and presence of the fish-exuded kairomone. Each treatment had ten replicates. Throughout the chronic experiments, the animals were fed everyday with the appropriate amount of pure *S. obliquus* culture to provide 1.0 mg C/L/individual (Vijverberg, 1989), which is above the incipient limiting level of carbon for *Daphnia* (Lampert, 1987).

In the former chronic toxicity experiment, neonates were exposed to increasing concentrations of NaCl, which were 0.00, 0.05, 0.10, 0.20, 0.40, 0.80 and 1.50 g/L, the absence (F-) and presence (F+) of the fish-exuded kairomone.

The latter chronic toxicity experiment was designed to determine the impacts of salinity on the life history traits of *Daphnia*, which were acclimated in a stepwise manner to several NaCl concentrations to mimic natural salt accumulation in real ecosystems. To obtain salt-acclimated individuals, daphnids that were already adapted to the COMBO culture medium, were transferred to COMBO media that

included 0.20 g/L of NaCl, which was the first main salinity level of the acclimation phase. *Daphnia* individuals were maintained at this salinity level for at least two weeks, which provided enough time to have at least two new generations (Vijverberg, 1989; Arnér, 1993; Baillieul *et al*., 1996; Martínez-Jerónimo and Martínez-Jerónimo, 2007). Some of these acclimated individuals were then transferred to media carrying the next level of salinity. This procedure was repeated up until test animals were acclimated to 0.20, 0.40, 0.60, 0.80, 1.10 g/L salinity levels. The acclimation procedure was carried out under the standard conditions mentioned above. Since the *D. pulex* individuals were not acclimated to fish-exuded kairomone, fish kairomone was not included as a stressor. After the acclimation phase, neonates were exposed to increasing concentrations of NaCl, which included 0.20, 0.40, 0.60, 0.80 and 1.20 g/L. In this experiment, the treatment containing 0.20 g/L NaCl was used as the control treatment according to the positive impact of 0.20 g/L NaCl on the growth of daphnids observed in previous experiments.

In both chronic experiments, daphnid survival was examined daily for three weeks. During the experiments, the test solutions were renewed every 3rd day in order to remove potentially interfering waste products and to reestablish the salinity stressor (Kashian and Dodson, 2002). There was no observable water loss likely due to evaporation. Additionally, in the chronic toxicity test with stepwise acclimation of *D. pulex* to salt, all the animals were photographed every second day. The aim of photographing, which was carried out with a camera that was attached to a microscope (Leica M Stereo Microscope - MZ16 and attached DFC280 Camera), was to obtain life history parameters – namely body length (distance from the top of the head to the base of the carapace spine), body width (distance taken from midpoint of the carapace), and egg number.

2.5 Sample Preparation for Spectral Studies

All of the sampled *D. pulex* individuals were frozen and stored at -4 °C until sample preparation for FTIR spectroscopic studies. These specimens were then lyophilized in a LABCONCO freeze dryer (FreeZone ®, Model 77520) overnight. An example photograph of dried *Daphnia magna* is presented in Figure 2.6-1. This minor dehydration step was carried out with the intention of removing any trace of intermolecular unbound water from the examined system so that the contribution of the broad absorption bands of water could be minimized.

2.6 ATR-FTIR Spectroscopy, Spectral Acquisition and Data Processing

The basic principle of Fourier Transform Infrared (FTIR) spectroscopy is that vibrations in molecules containing polar bonds are excited by infrared radiation at molecule-specific wavelengths dependent on the structural and atomic composition of the respective molecule (Freifelder, 1982; Stuart, 1997). ATR-FTIR spectroscopy (Attenuated Total Reflectance) is based on the measurement of infrared radiation reflectance from the sample. The penetration depth of infrared light in the sample for ATR measurements is independent of sample thickness and thus ATR spectroscopy requires minimal or no sample preparation prior to spectral measurements (Kazarian and Chan, 2006). Consequently, this approach was particularly suited to the examined daphnid system, where water would be a strong contributor to the infrared spectra.

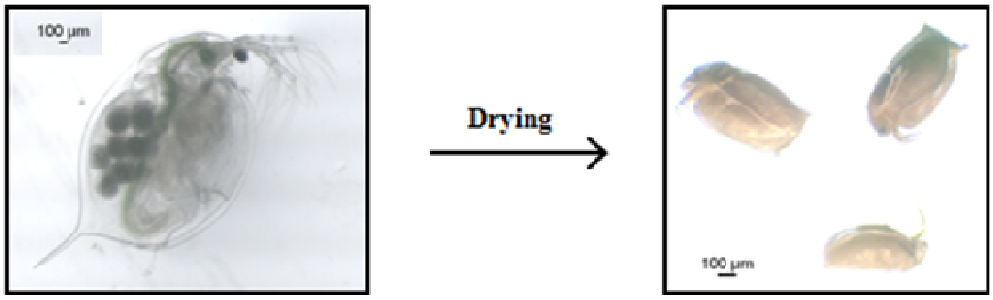

Figure 2.6-1. Photograph of freeze-dried *D. magna*.

Infrared spectra were generated using the one-bounce ATR mode in a Spectrum 100 Spectrometer (Perkin Elmer, Norwalk, CT, U.S.A.) fitted with a Universal ATR accessory. Each dried *D. pulex* individual was placed on a Diamond/ZnSe crystal plate one at a time and compressed (150 Gauge) to obtain good surface contact. Each sample was scanned with a spectral range between 4000-650 cm^{-1} at a resolution of 4 cm^{-1} with 250 scans at room temperature and ultimately one spectrum per daphnid individual was obtained.

In order to eliminate CO_2 and H_2O interference, background spectra were collected under identical conditions. These background spectra were subsequently subtracted from the sample spectra automatically via the Perkin Elmer Spectrum One software.

All digital data processing following the ATR-FTIR data acquisition from the control, cypermethrin-treated, and fish-exuded kairomone-exposed samples was performed with the Spectrum One software (Perkin Elmer). The quantitative assessment of the different functional groups was performed by the accurate detection of the peak area, which is expressed in arbitrary units, under the respective bands in the raw spectra. During the accurate determination of structural

variations, the peak positions were detected using the same software. The frequency corresponding to the center of weight of the spectra were calculated from spectra that were first smoothed with nine-point Savitsky-Golay smooth function to remove the noise.

On the other hand, in order to detect the changes in protein secondary structure, detailed analysis of the amide I band was carried out using the OPUS^{NT} data collection soft-ware package (Bruker Optics, Reinstetten, Germany). During this procedure, the second derivatives of the spectra that were first smoothed by applying a Savitzky-Golay algorithm with nine smoothing points were considered. These derivatives were further vector normalized in the 1700–1600 cm^{-1} spectral range. As a result, the peak intensities of the sub-bands of the amide I band were measured by recording the peak minima of the second derivative signals, as they are the points corresponding to the peak positions of original infrared spectra (Toyran *et al*., 2006; Simsek-Ozek *et al*., 2009). Spectra that were baseline-corrected and normalized with respect to specific bands were used for visual demonstration. The mean values and statistical analysis were performed accordingly.

2.7 Statistical Analyses

SAS-GLM Repeated Measures of 2-way ANOVA: (SAS System for AIX Version 5 Release 2). This statistical analysis allows for the determination of whether or not the singular and interactive time-dependent impacts of the experimental factors are statistically significant.

Probit Analysis: (EPA Probit Analysis Program Version 1.5) This analysis allows for the determination of the LC (lethal concentration) value according to the concentrations used in an acute toxicity experiment. Provided that the data were suitable, both the LC_{50} for 24 and 48 hrs can be calculated. In such studies, the LC_{50} (the concentration that causes the death of 50% of the population) value

calculated from the Probit analysis can be used during the determination of the concentrations to be used in a chronic toxicity experiment.

Dunnett's Pair-wise Multiple Comparison t-test: (SPSS 13.0 for Windows) Since it has a higher statistical power, Dunnett's t-test was used for pair-wise comparison. In order to standardize the survival data, the lifespan (days) of each individual was divided by the length of the experiment.

2.7.1 Cypermethrin Toxicity Experiments

Repeated-measures of two-way analysis of variance (rm-ANOVA) using General Linear Models was performed (SAS, 2002) to determine the effect of the treatments on survival throughout the duration of the experiment. To test the effect of cypermethrin and fish kairomone on clutch size and age at first reproduction, multivariate analysis of variance (MANOVA) was performed. The pair-wise comparison of the survival, clutch size and age at first reproduction parameters of the control (CM-) and cypermethrin-treated (CM+) samples of the treatments in the absence and presence of the fish-exuded kairomone in each experiment was made separately using Dunnett's pair-wise comparison test (CM- F- vs CM+ F- and CM- F+ vs CM+ F+).

During the detailed FTIR spectral analysis, prior to all statistical analyses, the Grubbs' test, also called the ESD method (extreme studentized deviate), was performed with the intention of determining whether or not one of the values in the list is a significant outlier from the rest. Significant outliers were removed from calculations. Subsequently, Dunnett's pair-wise comparison test was used in order to determine the effect of cypermethrin treatment on spectral parameters in both the absence and presence of the fish-exuded kairomone separately (CM- F- vs CM+ F- and CM- F+ vs CM+ F+).

For the comparison of the F- and F+ treatments in identical cypermethrin treatments, the Mann-Whitney U test was carried out. All MANOVA and pair-wise comparison analyses were performed using SPSS Statistitical Software Version 13.0 for Windows, SPSS Inc. (SPSS, 2004). Acceptable significance was recorded when p values were ≤ 0.05*.

2.7.2 Salinity Toxicity Experiments

EPA (Environmental Protection Agency) Probit Analysis Program (Version 1.5) was used to determine the 24 and 48hr LC_{50} (Lethal Concentration) in the acute salinity toxicity experiments. Repeated-measures of two-way analysis of variance (rm-ANOVA) using General Linear Models was performed (SAS, 2002) to determine the effect of both single and multiple stressors on survival. The effects of stressors within the chronic experiment with acclimated *D. pulex* individuals were performed using one-way ANOVA. Dunnett's Pair-wise Multiple Comparison test (SPSS 13.0 for Windows) was used for the pair-wise comparison of survival rates, clutch size, body width / body length ratio, and spectral parameters of control and NaCl-treated samples in the absence and presence of the fish-exuded kairomone, separately (NaCl- F- vs NaCl + F- and NaCl - F+ vs NaCl + F+). The comparison of the control (F-) and fish-conditioned (F+) treatments was made using the Mann-Whitney U test (SPSS 13.0 for Windows). Acceptable significance was recorded when p values were ≤ 0.05*.

CHAPTER 3

RESULTS

3.1 Effects of Pesticide and Fish Predation on *Daphnia pulex*: Organismal and Molecular Approach

The individual and interaction effects of cypermethrin and the chemical cue from fish on the survival, life history traits and molecular profile of *D. pulex* were investigated utilizing a joint approach of standard toxicity tests, which included both an acute and chronic tests, and infrared spectroscopy.

3.1.1 High-Concentration Acute Toxicity Test

Figure 3.1-1 shows the percent survival, while Table 3.1-1 presents the results of repeated measures (rm) of 2-way ANOVA applied to the survival data of the *D. pulex* individuals over time during an acute exposure to cypermethrin in the absence and presence of fish-exuded kairomone. No significant difference was observed between the survival of the individuals in the control and solvent control treatments in either the absence (F-) or the presence (F+) of the fish-exuded kairomone, suggesting that the concentration of acetone was not a factor affecting the response of daphnids to cypermethrin (CM). Figure 3.1-1 (F+ 0 µg/L and F+ Solvent) and Table 3.1-1 reveal that fish alone ($p > 0.05$) did not influence the survival of the daphnids, while cypermethrin alone ($p < 0.0001$***) reduced *Daphnia* survival significantly at concentrations of 1 and 5 µg/L. Moreover, the interaction of fish and cypermethrin was also significant ($p < 0.05$*) (Table 3.1-1). As can be seen from Figure 3.1-1, the interaction of CM and the fish kairomone at

a CM concentration of 1 µg/L was synergistic since the effect of 1 µg/L CM together with the fish kairomone on the mortality of *D. pulex* was greater than that of either treatment alone. In comparison to the absence of the fish kairomone, its presence resulted in greater mortality at a concentration of 1 µg/L CM, as can be clearly seen after 24 and 48 hrs. In other words, after 24 hrs of exposure to cypermethrin at concentrations of both 1 and 5 µg/L, at least 50% of the *D. pulex* population survived, even in the presence of the fish kairomone. However, after 48 hrs exposure 50% of the population of only the 1 µg/L CM lacking fish kairomone treatment (F- 1 µg/L) survived. *Daphnia* mortality was significantly greater at a concentration of 5 µg/L CM regardless of the presence of the fish kairomone.

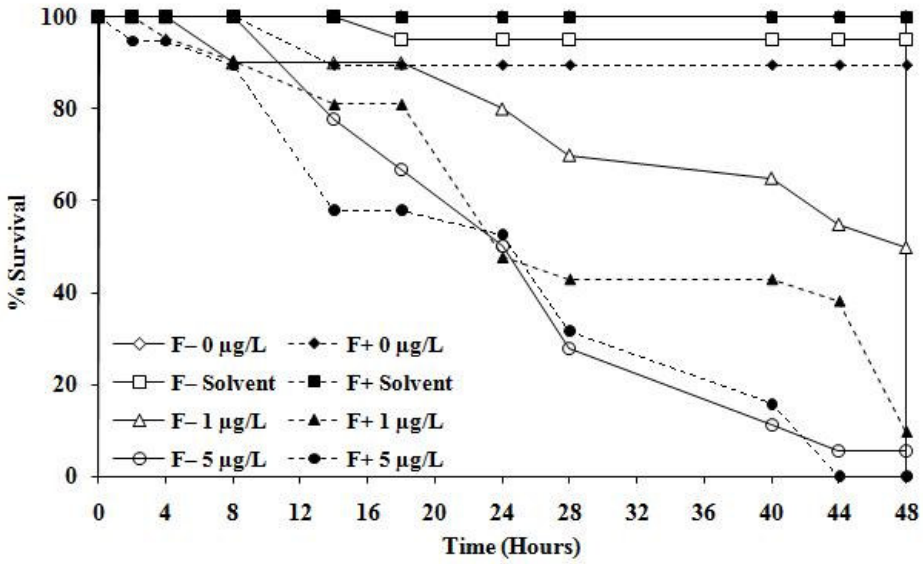

Figure 3.1-1. Percent survival of the *D. pulex* individuals over time during a high-concentration acute exposure to cypermethrin in the absence (F–) and presence (F+) of fish-exuded kairomone.

Table 3.1-1 Results of repeated measures of 2-way ANOVA applied to the survival data of *D. pulex* over time during high-concentration acute and intermediate-concentration and low-concentration chronic exposures to cypermethrin.

	p-value		
TREATMENT	**High (1.0-5.0 μg/L)**	**Intermediate (0.04-3.60 μg/L)**	**Low (0.5-15.0 ng/L)**
Fish	0.3636	0.0495*	0.6555
Cypermethrin	<.0001***	<.0001***	<.0001***
Fish*Cypermethrin	0.0448*	0.5429	0.9531

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

3.1.2 Intermediate-Concentration Chronic Toxicity Test

Figure 3.1-2 shows the percent survival, while Table 3.1-1 and Table 3.1-2 present the results of rm-2-way ANOVA and Dunnett's pair-wise comparison (CM- F- vs CM+ F- and CM- F+ vs CM+ F+) applied to the survival of the *D. pulex* individuals over time during an intermediate-concentration chronic exposure to cypermethrin in the absence and presence of fish-exuded kairomone. As expected, no significant effect of the solvent treatment was observed compared to the control treatment in either (F-) or (F+) conditions, supporting the previous suggestion that the concentration of acetone was not a critical factor affecting the daphnids. Figure 3.1-2 and Table 3.1-2 reveal that the survival of daphnids in the cypermethrin treatments decreased in a concentration dependent manner becoming statistically significant at concentrations higher than 0.30 μg/L ($p < 0.05$* or $p < 0.01$**) regardless of the presence of the fish kairomone. It is noteworthy that the statistical insignificance at concentrations lower than 0.30 μg/L was rather due to the slight mortality in acceptable ranges in the control treatments. However, it is clearly seen from the figure that at CM concentrations ≤ 0.90 μg/L the presence of the fish

kairomone increased the survival rate in comparison to its absence. This is coherent because the impact of the fish-exuded kairomone is significant ($p < 0.05$*), despite the fact that the interaction of fish*cypermethrin is non-significant ($p > 0.05$) (Table 3.1-1).

Table 3.1-2. Results of Dunnett's pair-wise comparison (CM- F- vs CM+ F- and CM- F+ vs CM+ F+) applied to the survival data of *D. pulex* over time during an intermediate-concentration chronic exposure to cypermethrin in the absence (F–) and presence (F+) of fish-exuded kairomone.

Comparison to	**0.00 µg/L**	
TREATMENT	**F–**	**F+**
Solvent + 0.00 µg/L	1.000	1.000
0.04 µg/L	.843	.998
0.10 µg/L	.870	.826
0.30 µg/L	.045*↓	.656
0.90 µg/L	.014*↓	.019*↓
1.80 µg/L	.017*↓	.005**↓
3.60 µg/L	.010**↓	.002**↓

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

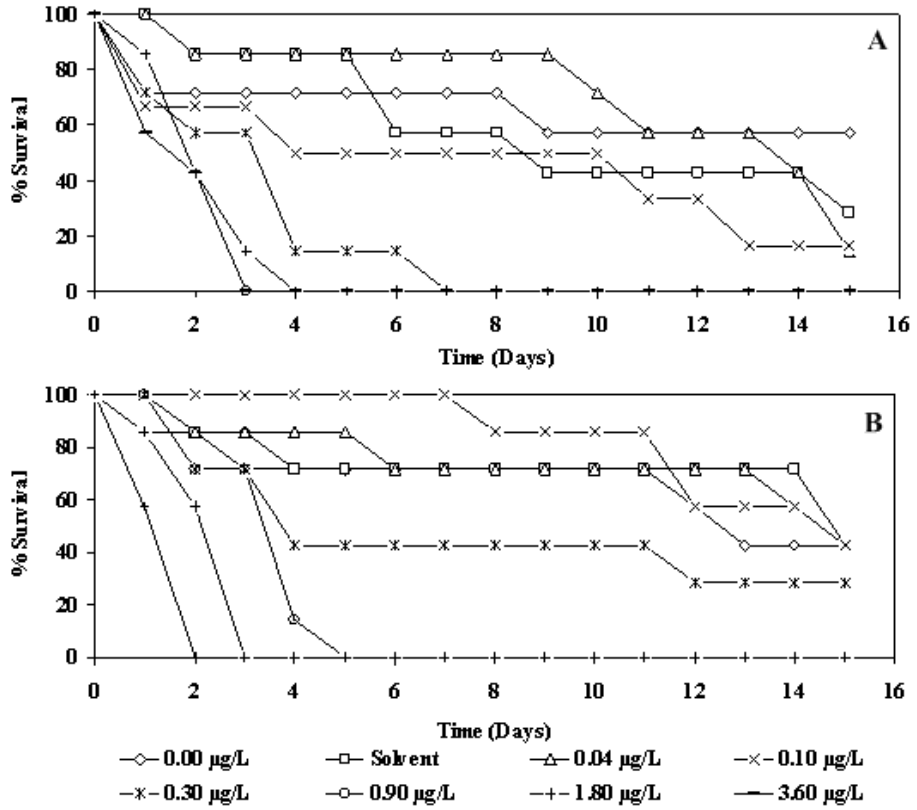

Figure 3.1-2. Percent survival of the *D. pulex* individuals over time during an intermediate-concentration chronic exposure to cypermethrin in the absence (A) and presence (B) of fish-exuded kairomone.

3.1.3 Low-Concentration Chronic Toxicity Test

Figure 3.1-3 shows the percent survival, while Table 3.1-1 and Table 3.1-3 present the results of rm-2-way ANOVA and Dunnett's pair-wise comparison (CM- F- vs CM+ F- and CM- F+ vs CM+ F+) applied to the survival data of *D. pulex* individuals over time during a low-concentration chronic exposure to cypermethrin in the absence and presence of fish-exuded kairomone. Figure 3.1-3 and Table 3.1-3 reveal that the survival of daphnids during low-concentration chronic exposure to cypermethrin decreased significantly at CM concentrations of 11.5 and 15.0 ng/L ($p < 0.001$***) regardless of the absence and presence of the fish kairomone ($p > 0.05$ for both fish and fish*cypermethrin treatments, Table 3.1-1).

Figure 3.1-4 shows the clutch size (fecundity) (A) and age at first reproduction (B), while Table 3.1-3 and Table 3.1-4 present the results of Dunnett's pair-wise comparison (CM– vs CM+) and MANOVA applied to the clutch size and age at first reproduction data of *D. pulex* individuals over time during the low-concentration chronic exposure to cypermethrin in the absence and presence of fish-exuded kairomone. Figure 3.1-4 and Table 3.1-3 reveal that the average clutch size decreased significantly ($p < 0.001$***) at CM concentrations of 11.5 and 15.0 ng/L, while the time for the first brood to appear (AFR) was prolonged at all CM concentrations. The presence of the fish-exuded kairomone slightly but not significantly reversed the impact of cypermethrin at low-concentrations by slightly increasing the average clutch size and slightly decreasing the age at first reproduction of the daphnids.

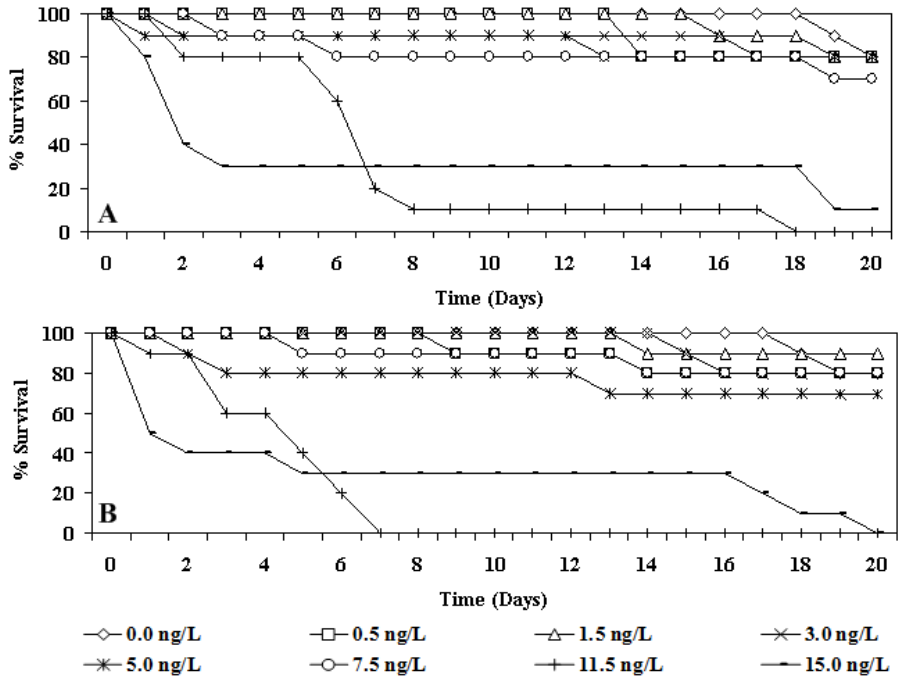

Figure 3.1-3. Percent survival of *D. pulex* individuals over time during a low-concentration chronic exposure to cypermethrin in the absence (A) and presence (B) of fish-exuded kairomone.

Table 3.1-3 Results of Dunnett's pair-wise comparison (CM- F- vs CM+ F- and CM- F+ vs CM+ F+) applied to the survival, clutch size and age at first reproduction (AFR) data of *D. pulex* over time during a low-concentration chronic exposure to cypermethrin in the absence (F–) and presence (F+) of fish-exuded kairomone.

	SURVIVAL		**CLUTCH SIZE**		**AFR**	
Comparison to			**0.0 ng/L**			
TREATMENT	**F–**	**F+**	**F–**	**F+**	**F–**	**F+**
0.5 ng/L	.997	.976	.999	.748	.086↑	.004**↑
1.5 ng/L	1.000	1.000	.976	.815	.000***↑	.000***↑
3.0 ng/L	.920	1.000	.948	.905	.005**↑	.017*↑
5.0 ng/L	.805	.275	.939	.590	.000***↑	.207
7.5 ng/L	.621	.919	.965	.825	.007**↑	.006**↑
11.5 ng/L	.000***↓	.000***↓	.000***↓	.000***↓	.906	-
15.0 ng/L	.000***↓	.000***↓	.000***↓	.000***↓	.090↑	.984

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

Table 3.1-4 Results of MANOVA applied to the clutch size and age at first reproduction (AFR) data of *D. pulex* during a low-concentration chronic exposure to cypermethrin.

TREATMENT	**CLUTCH SIZE**	**AFR**
Fish	0.171	0.004**
Cypermethrin	.000***	0.000***
Fish*Cypermethrin	.759	0.370

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

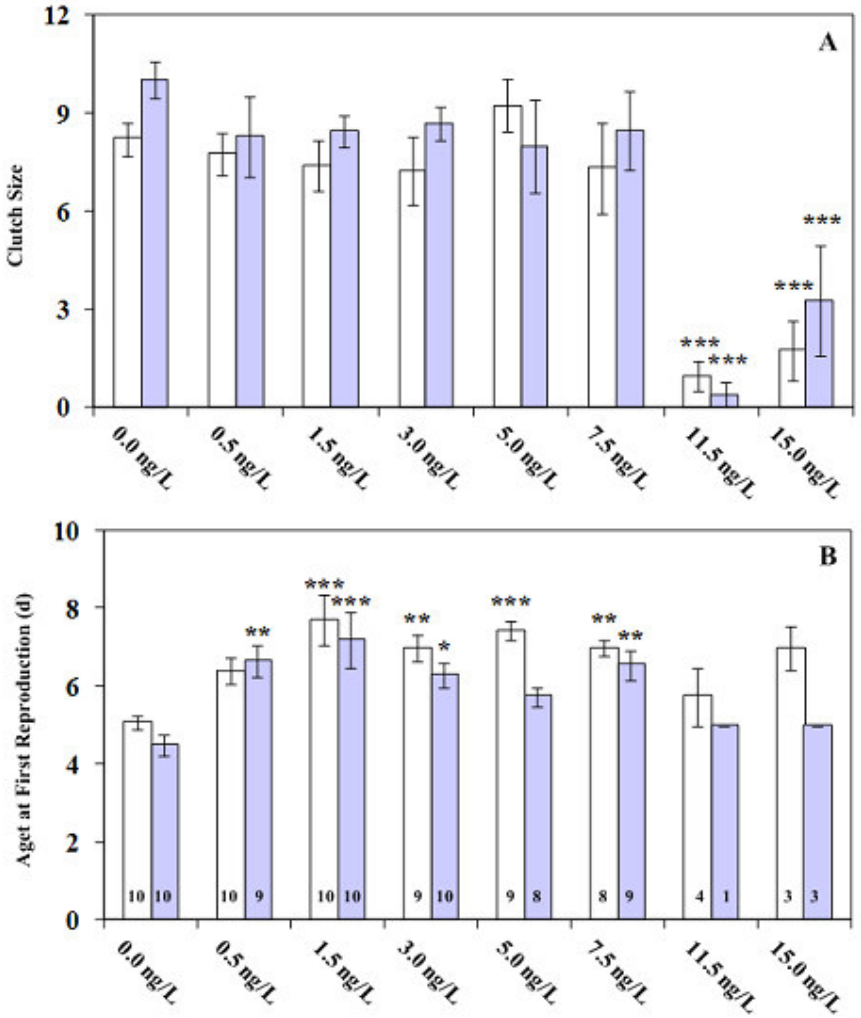

Figure 3.1-4. Clutch size (A) and time to first brood (AFR) (n values presented in bars) (B) of *D. pulex* individuals during a low-concentration chronic exposure to cypermethrin in the absence (open bars) and presence (shaded bars) of fish-exuded kairomone (Mean ± Std Error). Pair-wise comparison of control (CM-) and cypermethrin-treated (CM+) samples (CM- F- vs CM+ F- and CM- F+ vs CM+ F+) is determined according to the Dunnett's pair-wise comparison test (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

3.1.4 FTIR Spectroscopic Analyses

ATR-FTIR spectroscopy results in spectra that reflect the total biochemical composition of the examined system. Alterations in the frequency of infrared bands monitor structural variations; while variations in the signal intensity or more accurately the area under the peaks give information about the concentration of the respective functional groups (Cakmak *et al*., 2006; Bozkurt *et al*., 2007; Simsek-Ozek *et al*., 2009). Infrared spectra reveal the relative amounts of vibrationally active molecular groups (e.g. O-H, N-H, C=O, CH_2, CH_3, P=O, C-O-C) of major biomolecules, such as proteins, lipids, and carbohydrates in cells (Beardall *et al*., 2001; Dean *et al*., 2008).

Band Assignment – Molecular groups in organic samples are excited by electromagnetic radiation in the mid-infrared region (4000-400 cm^{-1}). Thus, spectral acquisition carried out on each *Daphnia pulex* individual generated infrared absorption spectra containing several clear bands over the wavenumber range 3800-650 cm^{-1}. Figure 3.1-5 shows the characteristic band profile of a representative ATR-FTIR spectrum of a lyophilized *D. pulex* individual from the control treatment (F- 0 µg/L) of the acute toxicity test. Table 3.1-5 shows the molecular assignments of the absorption bands, which have been labeled in this figure, based on literature sources on phytoplankton, bacteria, plants, fish and mammalian samples. In order to overcome the difficulty of interpreting the variations in these bands, where distinct molecular groups contribute to different bands and thus individual bands are due to more than one molecular source, the major contributor to a band is determined by seeking for correlations between bands of similar origins.

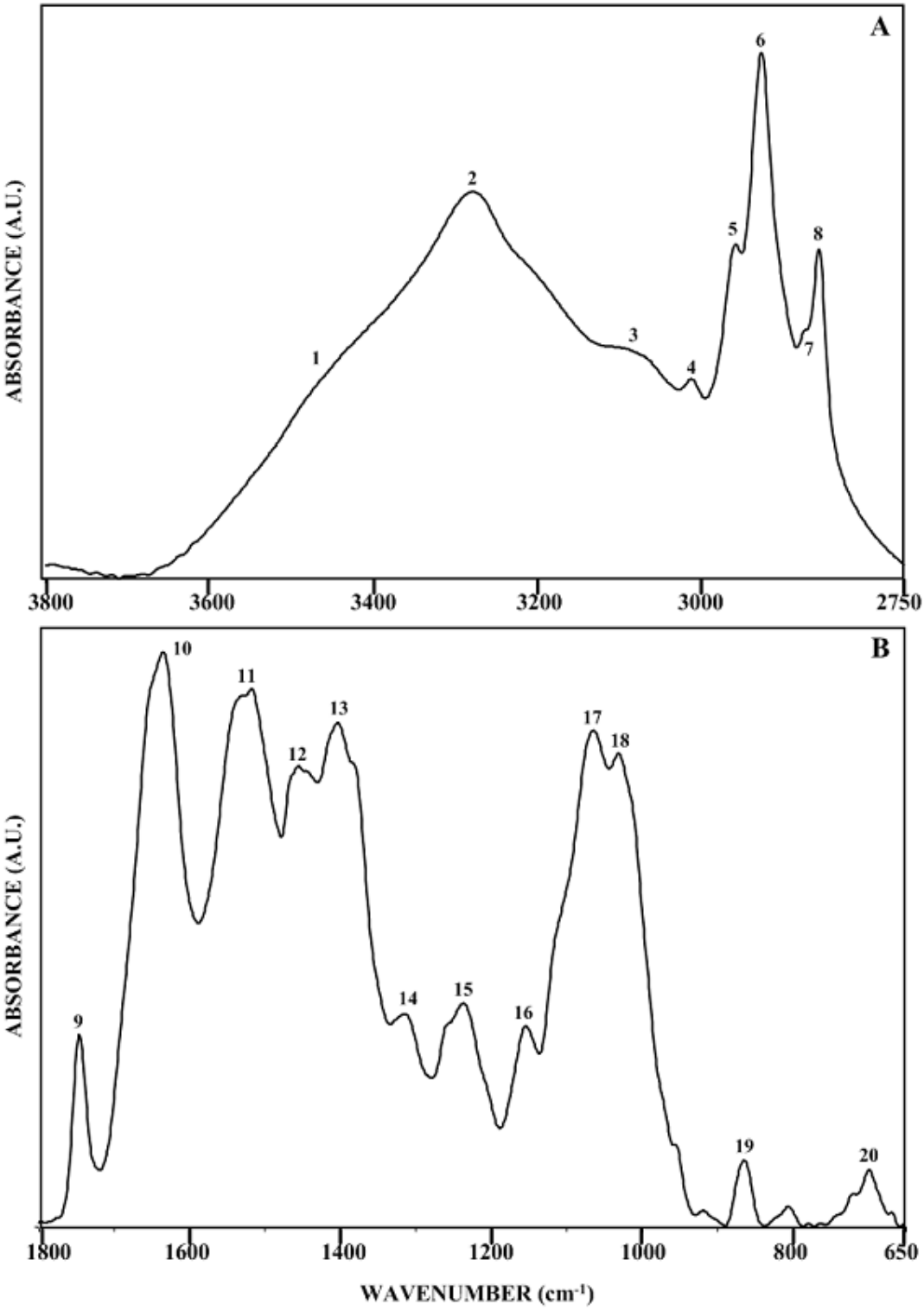

Figure 3.1-5. Representative ATR-FTIR spectrum of a *Daphnia pulex* individual in the (A) 3800-2750 cm^{-1} and (B) 1800-650 cm^{-1} frequency ranges.

The infrared spectral range between 3800-3100 cm^{-1} is dominated by broad spectral features due to N-H and O-H stretching modes (Maquelin *et al*., 2002; Sigee *et al*., 2002; Gorgulu *et al*., 2007). In this region it is characteristic for asymmetric and symmetric water stretching and N-H stretching vibrational bands to overlap (Garidel, 2003). However, in the current study, during sample preparation a minor dehydration step was carried out with the intention of removing any trace of free and unbound water from the examined daphnid system. The aim of this procedure is to minimize the contribution of the broad absorption bands of water to the spectra. However, during this procedure it was not aimed to remove all intra- and inter-molecular water, since complete dehydration alters the physical properties of biological systems. Nevertheless, a shoulder around 3450 cm^{-1} superimposed on the band located at 3297 cm^{-1} can be observed easily. Since this shoulder is due to intra-molecular hydrogen bonding, its presence confirms that the drying step carried out during this study was minor and thus intra- and inter-molecular hydrogen bonding remained in the examined system during freeze-drying (Simsek-Ozek *et al*., 2009). As a result, it can safely be stated that the band viewed at about 3297 cm^{-1} – denoted as the amide A band – is mainly due to the N-H and O-H stretching of polysaccharides and proteins (Akkas *et al*., 2007, Garip *et al*., 2009), with negligible contribution from the O-H stretching of intermolecular hydrogen bonding (Cakmak *et al*. 2006; Dogan *et al*. 2006). There is another band around 3080–3100 cm^{-1}, which is distinctive to weak N-H stretching absorptions, also referred to as the amide B band (3090 cm^{-1}) of protein fractions of any examined system (Garidel, 2003).

Table 3.1-5. Tentative assignment of bands found in the (A) 3800-2750 cm^{-1} and (B) 1800-650 cm^{-1} spectral regions of the ATR-FTIR spectra of lyophilized *Daphnia pulex* individuals.

PART A	Peak #	Wavenumber (cm^{-1})	Definition of the Spectral Assignment	References
Region		**3800-2750 cm^{-1} Spectral Region**		
3800-3100 cm^{-1} O-H and N-H Stretch	**1**	3450	Intra-molecular Hydrogen-bonding	Simsek-Ozek *et al*., 2009
	2	3297	Amide A: N-H and O-H stretching of polysaccharides and proteins	Sigee *et al*., 2002, Garidel, 2003, Garip *et al*., 2009
	3	3090	Amide B: C-N and N-H stretching of proteins	Garidel, 2003, Akkas *et al*., 2007, Simsek-Ozek *et al*., 2009
3100-2750 cm^{-1} C-H Stretch	**4**	3013	Olefinic HC=CH Stretch: Unsaturated lipids and cholesterol esters	Sigee *et al*., 2002, Akkas *et al*., 2007, Simsek-Ozek *et al*., 2009
	5	2958	CH_3 Asymmetric Stretch: Mainly lipids with a small contribution from proteins, carbohydrates, and nucleic acids	Akkas *et al*., 2007, Garip *et al*., 2009
	6	2924	CH_2 Asymmetric Stretch: Mainly lipids with a small contribution from proteins, carbohydrates, and nucleic acids	Beardall *et al*., 2001 Sigee *et al*., 2002, Gorgulu *et al*., 2007
	7	2874	CH_3 Symmetric Stretch: Mainly proteins with a small contribution from lipids, carbohydrates, and nucleic acids	Garip *et al*., 2009 Simsek-Ozek *et al*., 2009
	8	2853	CH_2 Symmetric Stretch: Mainly lipids with a small contribution from proteins, carbohydrates, and nucleic acids	Beardall *et al*., 2001 Sigee *et al*., 2002, Gorgulu *et al*., 2007

PART B	Peak #	Wavenumber (cm^{-1})	Definition of the Spectral Assignment	References
Region			**1800-650 cm^{-1} Spectral Region**	
	9	1733	C=O Stretch: Mainly esters of lipids and fatty acyl chains	Dean *et al*., 2007 Gorgulu *et al*., 2007 Mecozzi *et al*., 2007 Heraud *et al*., 2008
1700-1500 cm^{-1} Amide I and Amide II Groups	**10**	1642	Amide I: Mainly C=O stretching of proteins	Heraud *et al*., 2005 Stehfest *et al*., 2005 Mecozzi *et al*., 2007
	11	1527	Amide II: Mainly N-H bending and C-N stretching of proteins	Heraud *et al*., 2005 Stehfest *et al*., 2005 Cakmak *et al*., 2006
1450-1350 cm^{-1} C-H Bending and C-O Stretch	**12**	1449	CH_2 Asymmetric Bend: Lipids CH_2 and CH_3 Asymmetric Bend: Proteins	Sigee *et al*., 2002, Heraud *et al*., 2008 Dean *et al*., 2007 Garip *et al*., 2009
	13	1403	C-O Symmetric Stretch: COO^- groups of amino acid side chains and fatty acids CH_2 and CH_3 Symmetric Bend: Proteins	Sigee *et al*., 2002, Stehfest *et al*., 2005 Akkas *et al*., 2007 Dean *et al*., 2007
	14	1317	Amide III: N-H bending and C-N stretching of proteins	Heraud *et al*., 2005
1250-1200 cm^{-1} P=O Stretch	**15**	1238	PO_2^- Asymmetric Stretch: Phospholipids and nucleic acids	Heraud *et al*., 2005 Cakmak *et al*., 2006 Akkas *et al*., 2007 Mecozzi *et al*., 2007
1200-900 cm^{-1} C-O Stretch	**16**	1153	C-O Asymmetric Stretch: Polysaccharides	Sigee *et al*., 2002, Cakmak *et al*., 2006 Dean *et al*., 2007 Garip *et al*., 2009
	17	1069	PO_2^- Symmetric Stretch: Nucleic acids and phospholipids C-O Stretch: Polysaccharides	Sigee *et al*., 2002, Hirschmugl *et al*., 2006 Akkas *et al*., 2007 Dean *et al*., 2007
	18	1033	C-O Stretch: Polysaccharides	Beardall *et al*., 2001 Cakmak *et al*., 2006 Hirschmugl *et al*., 2006
900-650 cm^{-1} C-N Stretch	**19**	864	N-type Sugar	Garip *et al*., 2009
	20	699	CH_2 Bend: Carbohydrates, proteins and lipids	Mecozzi *et al*., 2007

A majority of C-H vibrations – namely methyl (CH_3) and methylene (CH_2) asymmetric and symmetric stretching vibrations and the olefinic HC=CH stretching mode – mainly due to lipids are recorded in the 3100-2800 cm^{-1} spectral range (Beardall *et al*., 2001; Sigee *et al*., 2002; Gorgulu *et al*., 2007; Simsek-Ozek *et al*., 2009). The 1730 cm^{-1} band corresponds to the C=O stretching vibration of the ester functional groups primarily belonging to lipids and fatty acids (Dean *et al*., 2007; Gorgulu *et al*., 2007; Mecozzi *et al*., 2007; Heraud *et al*., 2008). The 1700-1500 cm^{-1} region is composed of two major absorption bands mainly due to proteins. The first band, called amide I (1642 cm^{-1}), is described to be due to the C=O stretching vibrations, while the second band, termed amide II (1527 cm^{-1}), results from the combined vibrations of N–H bending and C–N stretching (Heraud *et al*., 2005; Stehfest *et al*., 2005; Cakmak *et al*., 2006; Mecozzi *et al*., 2007). Another amide band, which is also stated to be due to the N-H bending and C-N stretching of proteins, is observed at about 1317 cm^{-1} and is referred to as the amide III band (Heraud *et al*., 2005). The bands observed in the 1450-1350 cm^{-1} range are generally attributable to the asymmetric and symmetric bending vibrations of CH_3 and CH_2 groups likely in lipids and proteins (Cakmak *et al*., 2006; Dean *et al*., 2007; Heraud *et al*., 2008; Garip *et al*., 2009) in addition to the C-O symmetric stretching vibration of biomolecules containing COO- groups, such as amino acids and fatty acids (Heraud *et al*., 2005; Stehfest *et al*., 2005; Akkas *et al*., 2007; Garip *et al*., 2009). P=O asymmetric and symmetric stretching vibrations of phosphodiester groups of nucleic acids and phospholipids show absorbance between 1250-1200 cm^{-1} and 1085-1020 cm^{-1}, respectively (Cakmak *et al*., 2006; Dean *et al*., 2007; Mecozzi *et al*., 2007; Garip *et al*., 2009). While carbohydrates are the strongest absorbers in the 1200-900 cm^{-1} range, the P=O symmetric stretching vibration mentioned above displays an absorption band (1069 cm^{-1}) in the same region of the spectrum (Sigee *et al*., 2002; Hirschmugl *et al*., 2006; Akkas *et al*., 2007).

Nonetheless, absorbencies in this region are dominated by a complex sequence of superimposed peaks essentially due to C-O bonds (Sigee *et al*., 2002; Hirschmugl

et al., 2006; Akkas *et al*., 2007; Dean *et al*., 2007), such as those attributable to polysaccharides (Beardall *et al*., 2001; Giordano *et al*., 2001; Cakmak *et al*., 2006; Gorgulu *et al*., 2007). The region between 900 and 650 cm^{-1}, in which a variety of weak superimposed vibrations are present, is a spectral domain commonly referred to as the "fingerprint region" (Maquelin *et al*., 2002; Akkas *et al*., 2007; Garip *et al*., 2009). This region comprises absorption bands that are displayed by other classes of functional groups, such as C-N, originating from sugars and nucleic acids (Garip *et al*., 2009).

In the first part of the current study, the olefinic band (~3010 cm^{-1}) giving information about lipid unsaturation, the CH_2 asymmetric (~2925 cm^{-1}) and symmetric (~2855 cm^{-1}) bands characteristic of saturated lipids, the amide I band (~1634 cm^{-1}) providing information about the amide carbonyl stretching vibrations associated with proteins, and the CO-O-C asymmetric band (~1153 cm^{-1}) associated with carbohydrates like glycogen were investigated (Giordano *et al*., 2001; Cakmak *et al*., 2003; Garip *et al*., 2007).The alterations in the peak area values of the olefinic, CH_2 asymmetric + CH_2 symmetric, amide 1, and glycogen bands of *D. pulex* individuals chronically exposed to cypermethrin in both (F-) and (F+) conditions are given in Figure 3.1-6. The spectral results from both the intermediate- and low-concentration chronic toxicity tests indicated that the peak area of the olefinic band (~3010 cm^{-1}) had a general tendency to decrease with increasing cypermethrin concentration. A similar trend was observed for the alterations in the summation of the peak area values of the CH_2 asymmetric (CH_2 Asym) (~2925 cm^{-1}) and symmetric (CH_2 Sym) (~2855 cm^{-1}) bands. The decrease induced by CM at a concentration of 0.30 μg/L in the peak area values of both the olefinic and the (CH_2 Asym + CH_2 Sym) bands were slightly ($p < 0.05$*) counterbalanced in the presence of the fish-exuded kairomone. The CM-induced decrease was also valid for the peak area values of the amide I band (~1634 cm^{-1}) of the infrared spectra obtained from the *Daphnia* individuals after intermediate- and low-concentration chronic exposure in the absence and presence of fish-exuded kairomone.

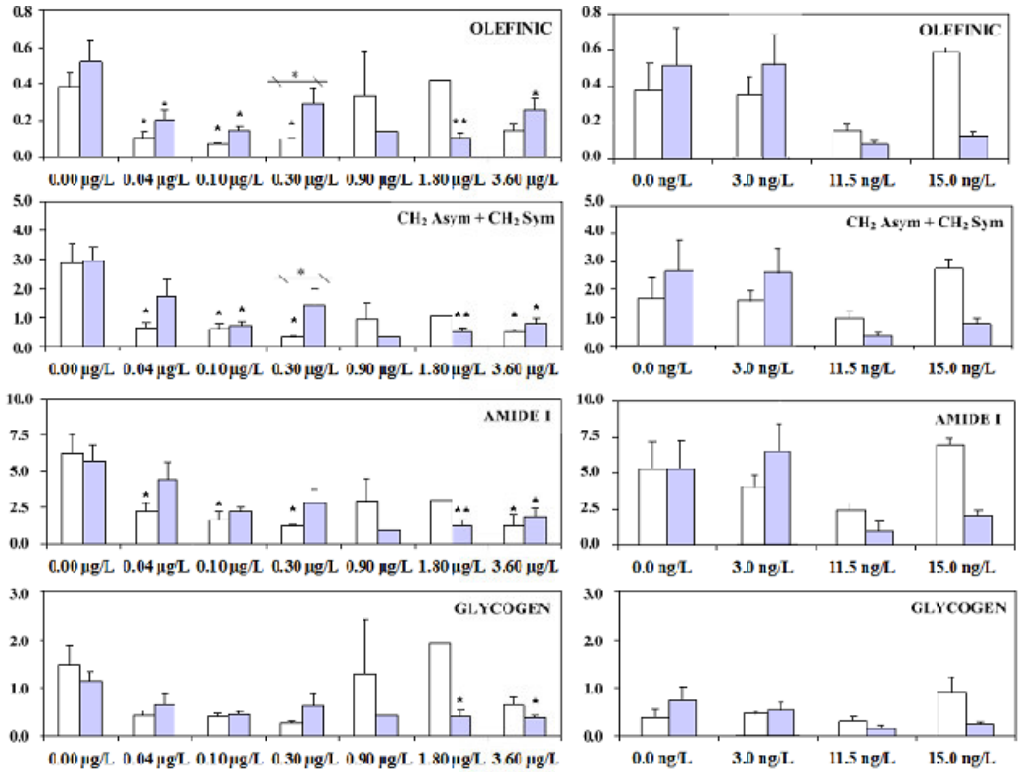

Figure 3.1-6. Peak area values of the olefinic, CH_2 Asymmetric (Asym) + CH_2 Symmetric (Sym), amide 1, and glycogen bands determined from the infrared spectra attained from *D. pulex* individuals (Left Panel: Intermediate-Concentration Chronic; Right Panel: Low-Concentration Chronic) exposed to cypermethrin in the absence (open bars) and presence (shaded bars) of fish-exuded kairomone (Mean ± Std Error). Comparison of control and treated samples (CM- F- vs CM+ F- and CM- F+ vs CM+ F+) is by means of the Dunnett's pair-wise comparison test, while comparison of the F- and F+ treatments in identical CM treatments is carried out via the Mann-Whitney U test (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

The general tendency of the direction of deviation of the CO-O-C asymmetric band (~1153 cm^{-1}) peak area in both the control and fish-conditioned treatments was also to decrease as the cypermethrin concentration increased. The presence of the fish-exuded kairomone did not have an effect on the peak area values determined for the low-concentration chronic toxicity test. The cypermethrin-induced decrease observed in the peak areas of the olefinic, (CH_2 Asym + CH_2 Sym), amide I and glycogen bands indicate a decrease in the concentration of lipids, proteins, and carbohydrates in the examined system.

3.2 Molecular Approach to the Effects of Pesticide and Fish Predation on *Daphnia*: An ATR-FTIR Spectroscopic Study

In this part of the study an approach linking individual level responses with molecular processes under more environmentally realistic conditions by means of higher-tier cypermethrin toxicity bioassays using the *Daphnia*-fish system complemented with ATR-FTIR spectroscopy was continued. However, detailed analysis of the ATR-FTIR spectroscopic measurements (see detailed explanation in section 3.1.4) were carried out in order to reveal information about the molecular profile of *Daphnia pulex* individuals exposed to cypermethrin and the fish-exuded kairomone simultaneously.

Acute Toxicity Test – The survival rate of the *D. pulex* individuals exposed to acute concentrations of cypermethrin in the absence and presence of the fish-exuded kairomone are presented in Figure 3.2-1. As observed from this figure, there was a significant decrease in the time of death of the daphnids as the concentration of cypermethrin increased.

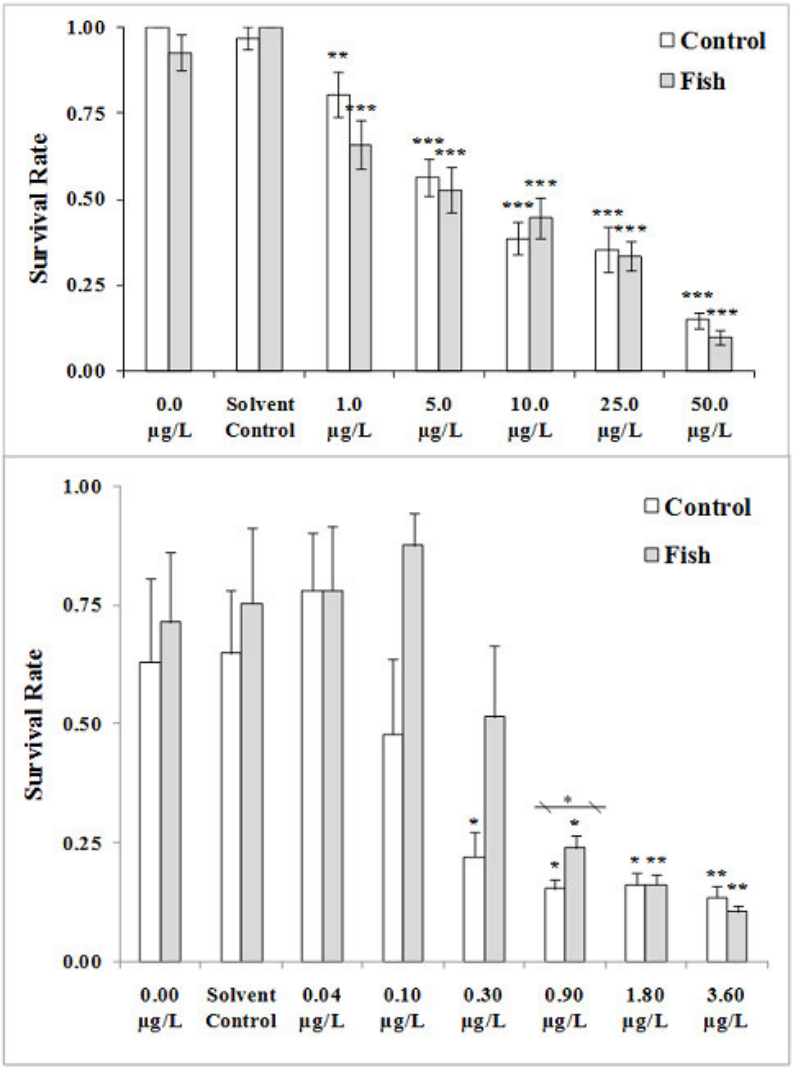

Figure 3.2-1. Survival rate of the *D. pulex* individuals over time during an acute (upper panel) and a chronic (lower panel) exposure to cypermethrin in the absence (F-) and presence (F+) of fish-exuded kairomone (Mean ± Std Error). Comparison of control and treated samples (CM- F- vs CM+ F- and CM- F+ vs CM+ F+) is by means of the Dunnett's pair-wise comparison test, while comparison of the F- and F+ treatments in identical CM treatments is carried out via the Mann-Whitney U test (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

It is worthy of note that an increase in the peak area values of the C=O ester band was observed regardless of the absence (F-) (Co: 0.057 ± 0.014; SCo: 0.065 ± 0.015 (NS); 1 µg/L: 0.093 ± 0.018 (NS); 5 µg/L: 0.144 ± 0.023 ($p < 0.05$*); 10 µg/L: 0.173 ± 0.028 ($p < 0.01$**); 25 µg/L: 0.183 ± 0.019 ($p < 0.001$***); 50 µg/L: 0.104 ± 0.023 (NS)) or presence (F+) (Co: 0.028 ± 0.004; SCo: 0.080 ± 0.014 ($p < 0.05$*); 1 µg/L: 0.064 ± 0.009 (NS); 5 µg/L: 0.062 ± 0.003 (NS); 10 µg/L: 0.122 ± 0.016 ($p < 0.001$***); 25 µg/L: 0.068 ± 0.010 (NS); 50 µg/L: 0.137 ± 0.008 ($p < 0.001$***)) of the fish-exuded kairomone. The peak area ratio of the ester C=O stretching vibration to the summation of the peak area values of the amide I and amide II bands were supportive of this cypermethrin concentration-dependent increase in C=O ester stretching vibration (data not shown).

Even though the time of exposure during this toxicity test was low (i.e. maximum 48 hrs), nevertheless there were some alterations in the molecular profile of the daphnids. When the *D. pulex* individuals were exposed to cypermethrin at acute concentrations (1.0-50.0 µg/L) in both the absence and presence of the fish-exuded kairomone, it was observed that the general tendency of the peak area values of the C-H stretching modes (3100-2750 cm^{-1}) – including their summation (CH_3 and CH_2 asymmetric and symmetric stretch) – attributed to lipids was to decrease. Similar tendencies were observed for the amide II band (1527 cm^{-1}) and the summation of the amide I (1642 cm^{-1}) and II bands (data not shown). These alterations were strengthened by the precise lipid-to-protein ratio calculated from the peak area values of the above stated vibrational modes. In the absence of the fish-exuded kairomone, the lipid-to-protein ratios measured by calculating the ratio of the areas of (CH_2 asymmetric stretch + CH_2 symmetric stretch) to CH_3 symmetric stretch increased with increasing cypermethrin concentrations.

To be precise, the peak area values of the bands arising from lipids and proteins individually showed tendencies to decrease, but their ratio to each other increased significantly (Table 3.2-1). This indicates that the cypermethrin-induced decrease in protein content was more pronounced than the decrease in the amount of lipids.

However, when the fish-exuded kairomone was present, the lipid-to-protein ratio calculated in a similar manner decreased with increasing cypermethrin concentrations (Table 3.2-1). This observation is the sign of a more pronounced decrease in the amount of lipids in comparison to proteins. The lipid-to-protein ratios measured by calculating the ratio of the areas of (i) (CH_2 asymmetric stretch + CH_2 symmetric stretch) to amide I and (ii) CH_3 asymmetric stretch to CH_3 symmetric stretch also decreased from 0.3853 ± 0.0168 to 0.1685 ± 0.0067 ($p<0.001$***) and 3.6858 ± 0.0533 to 3.1191 ± 0.1814 ($p<0.01$**), respectively, between (F+ 0µg/L) and (F+ 50µg/L) in a concentration-dependent manner. These decreases are consistent with the above mentioned decrease in the lipid-to-protein ratio in the daphnids exposed to increasing cypermethrin concentrations in the presence of the fish-exuded kairomone.

Chronic Toxicity Test – In order to investigate the above mentioned molecular alterations observed in the infrared spectra of the daphnids acutely exposed to cypermethrin in the absence and presence of the fish-exuded kairomone in more detail, a chronic toxicity test with a cypermethrin range of 0.04-3.60 µg/L in the absence (F-) and presence (F+) of the fish-exuded kairomone was designed.

As observed from Figure 3.2-1, the survival rate of the *D. pulex* individuals exposed to chronic concentrations of cypermethrin starts to decrease significantly at cypermethrin concentrations ≥ 0.30 µg/L in the absence of the fish-exuded kairomone and at cypermethrin concentrations ≥ 0.90 µg/L presence of the fish-exuded kairomone. Additionally, the effect of the presence of the fish-exuded kairomone is antagonistic at cypermethrin concentrations 0.10-0.90 µg/L, above which are levels more equivalent to the cypermethrin concentrations used in the acute toxicity test. For the details of the individual level responses please see section 3.1.

Table 3.2-1. Changes in the ratio values of the areas of (CH_2 asym str + CH_2 sym str) to CH_3 sym str from the ATR-FTIR spectra attained from *D. pulex* individuals exposed to (A) acute and (B) chronic levels of cypermethrin (Mean ± Std Error). Comparison carried out to determine the effect of CM in the absence or presence (shaded column) of the fish-exuded kairomone separately, is according to the Dunnett's pair-wise comparison test (CM- F- vs CM+ F- and CM- F+ vs CM+ F+), while the comparison of the F- and F+ treatments in identical CM treatments is performed with the Mann-Whitney U test (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

PART A		
ACUTE DOSES (µg/L)		**(CH_2 Asym Str + CH_2 Sym Str) / (CH_3 Sym Str)**
Control	**F-**	5.144±0.119
	F+	9.657±0.086
	F- vs F+	***↑
SCo	**F-**	4.708±0.047↓
	F+	6.226±0.049***↓
	F- vs F+	***↑
1.0	**F-**	7.151±0.091***↑
	F+	5.560±0.090***↓
	F- vs F+	***↓
5.0	**F-**	6.650±0.070***↑
	F+	6.330±0.124***↓
	F- vs F+	NS
10.0	**F-**	8.488±0.180***↑
	F+	5.398±0.070***↓
	F- vs F+	***↓
25.0	**F-**	5.535±0.076↑
	F+	4.700±0.063***↓
	F- vs F+	***↓
50.0	**F-**	4.879±0.084
	F+	5.214±0.086***↓
	F- vs F+	**↑

PART B		
CHRONIC DOSES (µg/L)		**(CH_2 Asym Str + CH_2 Sym Str) / (CH_3 Sym Str)**
Control	**F-**	9.620±0.320
	F+	9.073±0.307
	F- vs F+	NS
SCo	**F-**	9.612±0.204
	F+	6.331±0.056***↓
	F- vs F+	*↓
0.04	**F-**	6.474±0.124***↓
	F+	7.995±0.285**↓
	F- vs F+	**↑
0.10	**F-**	7.790±0.215***↓
	F+	8.664±0.280↓
	F- vs F+	*↑
0.30	**F-**	5.270±0.093***↓
	F+	8.589±0.214↓
	F- vs F+	**↑
0.90	**F-**	7.121±0.249***↓
	F+	7.433
	F- vs F+	NS
1.80	**F-**	7.332
	F+	5.782±0.082***↓
	F- vs F+	NS
3.60	**F-**	7.823±0.076***↓
	F+	5.274±0.050***↓
	F- vs F+	NS

The peak area values of the infrared peaks of *D. pulex* individuals exposed to chronic levels of cypermethrin in both the absence and presence of the fish-exuded kairomone are listed in Table 3.2-2. As can be observed from the table, there were pronounced reductions in the area values of the peaks in the 3100-2750 cm^{-1} spectral region, which is populated by absorptions arising from C-H stretching modes of =CH, $–CH_2$, and $–CH_3$ groups. The final reduction in the 3.60 µg/L cypermethrin treatment in comparison to the control treatment was 62%, regardless of the absence or presence of the fish-exuded kairomone, for the olefinic HC=CH stretching band. The final reduction in the 3.60 µg/L CM-treatment from the control treatment in the absence (F-) of the fish kairomone for the CH_3 asymmetric, CH_2 asymmetric, CH_3 symmetric, and CH_2 symmetric stretching vibrations was 71, 81, 79, and 87%, respectively. However, the final reduction determined in a similar manner (F+ 0.00 µg/L vs F+ 3.60 µg/L) for the treatments mimicking the presence of the fish kairomone was 50, 73, 55, and 73%, respectively. Supportive of the decreases observed for the C-H stretching vibrations attributed to lipids, the peak area values of the ester C=O and PO_2^- asymmetric stretching modes in the 3.60 µg/l cypermethrin treatment also showed decreases relative to the control treatment. This reduction was by 95% (F-) and 93% (F+) in the C=O stretching band and 69% (F-) and 66% (F+) in the PO_2^- asymmetric stretching band. The peak area ratio presented in Table 3.2-1 strengthens the observations made for the peak areas. The lipid-to-protein ratio measured by calculating the ratio of the areas of (CH_2 asymmetric stretch + CH_2 symmetric stretch) to CH_3 symmetric stretch decreased with increasing cypermethrin concentrations in both F- and F+ conditions. This indicates that the decrease in lipids (CH_2 asymmetric stretch + CH_2 symmetric stretch) is more pronounced than the decrease in proteins (CH_3 symmetric stretch) and thus results in a decrease in the lipid-to-protein ratio. This decrease in lipid-to-protein ratio due to a more marked decrease in the lipid content during the presence of the fish-exuded kairomone resembles the alterations in the acute toxicity test.

When the impact of the presence of the fish-exuded kairomone is compared with its absence in the case of the lipid-to-protein ratio ((CH_2 asymmetric stretch + CH_2 symmetric stretch) / CH_3 symmetric) (Table 3.2-1), it can be observed that the decrease induced by cypermethrin alone (F-) is counterbalanced by the fish-exuded kairomone by significant increases in cypermethrin concentrations between 0.04-0.90 µg/L. This is an observation that supports the antagonistic effect of the fish-exuded kairomone that was suggested from the survival data (Figure 3.2-1). Moreover, the general trend of the wavenumber values of the CH_3 asymmetric (2958 cm^{-1}) band was to shift to higher frequencies in a concentration dependent manner, regardless of the fish-exuded kairomone (Table 3.2-3).

The peak area values of the C-O asymmetric stretching (1153 cm^{-1}) and C-O stretching (1033 cm^{-1}) bands, which are two bands that are generally attributed to polysaccharides, showed decreases in response to cypermethrin exposure. This reduction was by 55% (F-) and 67% (F+) in the C-O band positioned at 1153 cm^{-1} and 20% (F+) and 58% (F-) in the C-O band located at 1033 cm^{-1} (Table 3.2-2).

Table 3.2-2. Changes in the peak area values of the bands found in the (A) 3800-2750 cm^{-1} and (B) 1800-650 cm^{-1} spectral regions of the ATR-FTIR spectra attained from *D. pulex* individuals exposed to chronic levels of cypermethrin (Mean ± Std Error). Comparison carried out to determine the effect of CM in the absence (open column) or presence (shaded column) of the fish-exuded kairomone separately, is according to the Dunnett's pair-wise comparison test (CM- F- vs CM+ F- and CM- F+ vs CM+ F+), (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

Peak #		4	5	6	7	8
Spectral Assignment		Olefinic HC=CH Str	CH_3 Asym Str	CH_2 Asym Str	CH_3 Sym Str	CH_2 Sym Str
Control	F-	0.383±0.074	0.931±0.185	2.035±0.463	0.297±0.066	0.829±0.187
	F+	0.520±0.114	0.960±0.187	2.134±0.351	0.328±0.058	0.806±0.129
SCo	F-	0.249±0.086	0.500±0.192	1.045±0.498	0.149±0.068	0.428±0.205
	F+	0.054±0.005 ***↓	0.167±0.023*↓	0.223±0.046**↓	0.047±0.009**↓	0.072±0.015*↓
0.04 µg/L	F-	0.107±0.027 *↓	0.291±0.078*↓	0.468±0.127*↓	0.098±0.026*↓	0.179±0.047*↓
	F+	0.204±0.050 *↓	0.701±0.191	1.192±0.410	0.207±0.063	0.533±0.175
0.10 µg/L	F-	0.066±0.012 *↓	0.242±0.059*↓	0.400±0.135*↓	0.074±0.022*↓	0.184±0.054*↓
	F+	0.143±0.022 **↓	0.304±0.052*↓	0.513±0.095*↓	0.087±0.016*↓	0.219±0.038↓
0.30 µg/L	F-	0.094±0.008 ↓	0.144±0.012*↓	0.266±0.009*↓	0.069±0.002*↓	0.090±0.002*↓
	F+	0.295±0.074	0.501±0.147	1.004±0.379↓	0.161±0.056	0.445±0.162
0.90 µg/L	F-	0.335±0.243	0.605±0.438*↓	0.747±0.482	0.128±0.078	0.181±0.103↓
	F+	0.139	0.230	0.264	0.045	0.071
1.80 µg/L	F-	0.416	0.665	0.811	0.141	0.219
	F+	0.083±0.014***↓	0.313±0.072	0.371±0.098**↓	0.087±0.023*↓	0.140±0.040**↓
3.60 µg/L	F-	0.145±0.028	0.267±0.039	0.381±0.064↓	0.062±0.011↓	0.105±0.023*↓
	F+	0.197±0.041 *↓	0.479±0.113	0.578±0.130**↓	0.149±0.032	0.214±0.046*↓

Table 3.2-2. Continued.

PART B	Peak #	9	10	11	15	16	17	18
Spectral Assignment		C=O Stretch	Amide I	Amide II	PO_2^- Asym Str	C-O Asym Str	PO_2^- Sym Str	C-O Stretch
Control	F-	0.585±0.192	6.200±1.277	5.380±1.162	2.340±0.528	1.472±0.395	2.629±0.524	2.195±0.512
	F+	0.546±0.086	5.698±1.100	6.637±0.657	2.044±0.397	1.132±0.204	3.499±0.732	3.661±0.288
SCo	F-	0.098±0.051*↓	3.716±1.356	2.844±1.095***↓	1.347±0.479	0.844±0.328	2.035±0.675**↓	1.533±0.539
	F+	0.043±0.006**↓	0.762±0.233**↓	0.629±0.202 **↓	0.312±0.088*↓	0.108±0.039**↓	0.318±0.092**↓	0.300±0.099***↓
0.04 µg/L	F-	0.112±0.026	2.262±0.586*↓	1.759±0.471*↓	0.935±0.250	0.431±0.105	0.856±0.209	0.607±0.141↓
	F+	0.163±0.086*↓	4.392±1.267	4.995±1.228	1.563±0.467	0.666±0.196	2.325±0.504	2.246±0.470
0.10 µg/L	F-	0.087±0.040*↓	1.626±0.563*↓	1.157±0.380*↓	0.667±0.189↓	0.412±0.068	0.767±0.167	0.618±0.076
	F+	0.071±0.013*↓	2.243±0.268	1.845±0.373**↓	0.775±0.080	0.446±0.061	1.098±0.127*↓	1.072±0.140**↓
0.30 µg/L	F-	0.077±0.004*↓	1.279±0.096*↓	0.825±0.145**↓	0.354±0.116*↓	0.213±0.069*↓	0.537±0.108	0.507±0.112↓
	F+	0.241±0.117↓	2.830±0.882	2.416±0.773**↓	1.228±0.380	0.648±0.212	1.584±0.508*↓	1.518±0.510**↓
0.90 µg/L	F-	0.017±0.012*↓	2.958±1.506	0.465±0.196*↓	1.253±0.996	1.299±1.141	3.966±2.763	2.829±1.676
	F+	0.012	0.955	0.249	0.206	0.434	1.529	1.169
1.80 µg/L	F-	-	2.985	0.264	1.093	1.897	5.799	4.130
	F+	0.026±0.019***↓	1.212±0.434**↓	1.154±0.508***↓	0.470±0.077*↓	0.397±0.141*↓	0.749±0.126**↓	1.268±0.292**↓
3.60 µg/L	F-	0.029±0.018*↓	1.264±0.773*↓	0.178±0.250**↓	0.718±0.337	0.660±0.147	1.983±0.007	1.749±0.390
	F+	0.033±0.019**↓	1.916±0.510*↓	1.840±0.496**↓	0.697±0.186	0.378±0.062*↓	1.740±0.297	1.522±0.280*↓

Table 3.2-3. Changes in the wavenumber values of the amide A and CH_3 asymmetric stretch bands from the ATR-FTIR spectra attained from *D. pulex* individuals exposed to chronic levels of cypermethrin (Mean ± Std Error). Comparison carried out to determine the effect of CM in the absence (open column) or presence (shaded column) of the fish-exuded kairomone separately, is according to the Dunnett's pair-wise comparison test (CM- F- vs CM+ F- and CM- F+ vs CM+ F+) (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

CHRONIC DOSES (µg/L)		Amide A	CH_3 Asymmetric
Control	F-	3287.513±2.827	2956.41±0.113
	F+	3281.959±4.472	2956.227±0.106
SCo	F-	3295.307±2.513	2957.287±0.47
	F+	3311.916±8.601	2958.671±0.729
0.04	F-	3297.443±1.553	2957.327±0.315
	F+	3295.241±4.364	2958.752±1.262
0.10	F-	3302.893±9.413	2956.451±0.087
	F+	3298.659±7.108	2958.155±0.947
0.30	F-	3311.841±2.115*↑	2957.115±0.154
	F+	3312.61±8.003↑	2959.545±1.306
0.90	F-	3323.19±1.93**↑	2959.978
	F+	3363.305	-
1.80	F-	3337.4060	2966.407
	F+	3337.729±11.639***↑	2962.764±2.389*↑
3.60	F-	3355.297±5.781***↑	2960.588±1.337***↑
	F+	3338.105±11.279***↑	2963.811±1.981**↑

Last but not least; it can be observed from Table 3.2-2 that there are significant impacts on the peak area values of the FTIR spectra attained from the *D. pulex* individuals exposed to the solvent control treatment. This observation is exceptionally true in the treatments, where the fish-exuded kairomone is present.

Protein Secondary Structure Determination – One of the major strengths of infrared spectroscopy is that it allows the detection of relative differences in

secondary and tertiary structure induced by external factors, in this case exposure to abiotic and biotic stress factors (Jackson and Mantsch, 1995). Polypeptide and protein repeat units give rise to several characteristic infrared bands, of which the amide I band (1700-1600 cm^{-1}) is the most sensitive to the protein secondary structural components because it is almost entirely due to the C=O stretch vibrations of the peptide linkages. On the other hand, the amide II band shows much less sensitivity to protein conformation than its amide I counterpart because of its nature combining both N-H bending and C-N stretching vibrations (Krimm and Bandekar, 1986; Kong and Yu, 2007). Therefore, the intensities of the sub-components of the amide I band, which are closely correlated to each secondary structural element of proteins, were observed in the current study (Table 3.2-4). The sub-component located between 1695-1670 cm^{-1} is mainly due to antiparallel-aggregated β-sheet structure. However, this component is normally weak and so the overlapping absorptions from turn and unordered structures make its precise assignment quite difficult. The peak between 1669 and 1659 cm^{-1} arises from turn structures, while the 1658-1650 cm^{-1}range can be assigned to α-helical structure with some overlap with random coil structure. Random coil structures lead to band in the 1647-1641 cm^{-1} spectral region, while the peak in between 1640-1630 cm^{-1} corresponds to β-sheet structures. The band located between 1629-1620 cm^{-1} is attributed to aggregated β-sheet structure (Jackson and Mantsch, 1995; Haris and Severcan, 1999; Jung, 2000; Souillac *et al*., 2002; Stuart, 1997; Toyran *et al*., 2006; Kong and Yu, 2007; Simsek-Ozek *et al*., 2009). It is of note that the 1618-1605 cm^{-1} component is too low to be assigned to any secondary structure and thus likely arises from protein side chains (Zhang *et al*., 1998).

The intensities of the assigned sub-peaks of the amide I band of the exposed *Daphnia* individuals are presented in Figure 3.2-2. According to these results, it can be observed that both acute and chronic exposures to cypermethrin resulted in an increase in antiparallel β-sheet structure and a decrease in β-sheet structures. Chronic exposure also resulted in a decrease in the contribution of turns to the protein system. These cypermethrin-induced conformational changes in proteins

were further emphasized by the observed decrease in the peak area values of the amide I and amide II band (Table 3.2-2). The wavenumber of the amide A band showed a shift to higher frequencies when exposed to cypermethrin in both the absence and presence of the fish-exuded kairomone (Table 3.2-3). This increase in the frequency of the amide A band indicates alterations in the conformational structure of proteins, and is thus consistent with the alterations observed in the second derivative spectra of the *D. pulex* individuals exposed to cypermethrin in the absence and presence of the fish-exuded kairomone.

Table 3.2-4. Tentative assignment of the main protein secondary structural constituents of the vector normalized second derivative of the amide I band found in the ATR-FTIR spectra of lyophilized *Daphnia pulex* individuals (Jackson and Mantsch, 1995; Haris and Severcan, 1999; Jung, 2000; Souillac *et al*., 2002; Stuart, 1997; Toyran *et al*., 2006; Kong and Yu, 2007; Simsek-Ozek *et al*., 2009).

Peak #	Wavenumber (cm^{-1})	Assignment
1	1695-1670	Antiparallel-Aggregated β-sheet
2	1669-1659	Turns
3	1658-1650	α-helix
4	1647-1641	Random coil
5	1640-1630	β-sheet
6	1629-1620	Aggregated β-sheet
7	1618-1605	Side chains

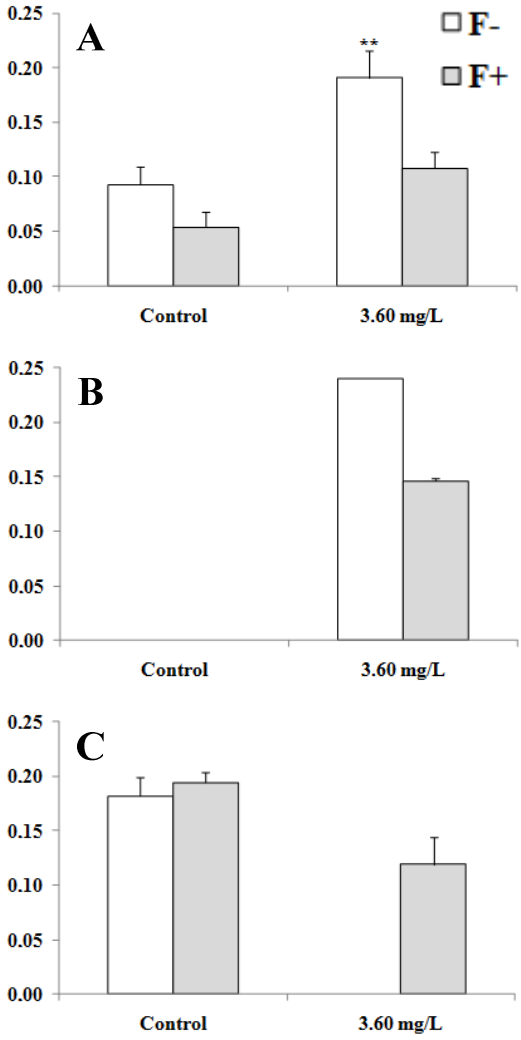

Figure 3.2-2. Changes in the intensities of the sub-bands corresponding to (A) antiparallel-aggregared β-sheet, (B) random coil, and (C) β-sheet structures of the vector normalized second derivative of the amide I band found in the ATR-FTIR spectra attained from *D. pulex* individuals exposed to chronic levels of cypermethrin (Mean ± Std Error). Comparison carried out to determine the effect of CM in the absence (open column) or presence (shaded column) of the fish-exuded kairomone separately, is according to the Dunnett's pair-wise comparison test (CM- F- vs CM+ F- and CM- F+ vs CM+ F+) (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

3.3 Impacts of Salinity and Fish-Exuded Kairomone on the Survival and Molecular Profile of *Daphnia*

The present part of this thesis was carried out in order to investigate the separate and interactive effects of NaCl salinity and the fish-exuded kairomone at the organismal and molecular levels of *D. pulex* utilizing standard acute and chronic toxicity tests (Bezirci, 2008) that were complemented with infrared spectroscopy.

3.3.1 Acute Toxicity Experiments

As there has not been any investigation to study the combined effects of salinity and fish-exuded kairomone on survival of *D.pulex*, the first acute toxicity experiment had to include a wide range of concentrations of NaCl exposed simultaneously with the fish-exuded kairomone to be able to develop reliable concentrations for chronic exposures.

It was observed that, increasing salt concentration, fish-exuded kairomone and time had significant impacts on the survival of daphnids ($p < 0.001$***, Table 3.3-1, Figure 3.3-1). Up to 4.00 g/L of salinity, the presence of fish-exuded kairomone reversed the salinity-induced significant decrease in survival, while this effect of the kairomone disappeared and daphnid survival further decreased significanty at salinity concentrations ≥ 6.00 g/L (Table 3.3-1, Figure 3.3-1). These data could not be analyzed using Probit analyses because the survival of the individuals were too low. However, these data were utilized for determining the concentrations of the second acute toxicity experiment.

Table 3.3-1. Results of repeated measures of 2-way ANOVA applied to the survival data of *D. pulex* over time during two acute and one chronic exposure to salinity.

		Acute		Chronic
	Treatment	**First**	**Second**	
Impact	**Fish**	0.0002***	0.0066***	0.8796
	Salinity	<.0001***	<.0001***	<.0001***
	Time	<.0001***	<.0001***	<.0001***
Interaction	**Fish*Salinity**	0.0007***	<.0001***	0.7710
	Fish*Time	0.0580	0.3242	0.4236
	Salinity*Time	<.0001***	<.0001***	<.0001***
	Fish*Time*Salinity	<.0001***	1.0000	<.0001***

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

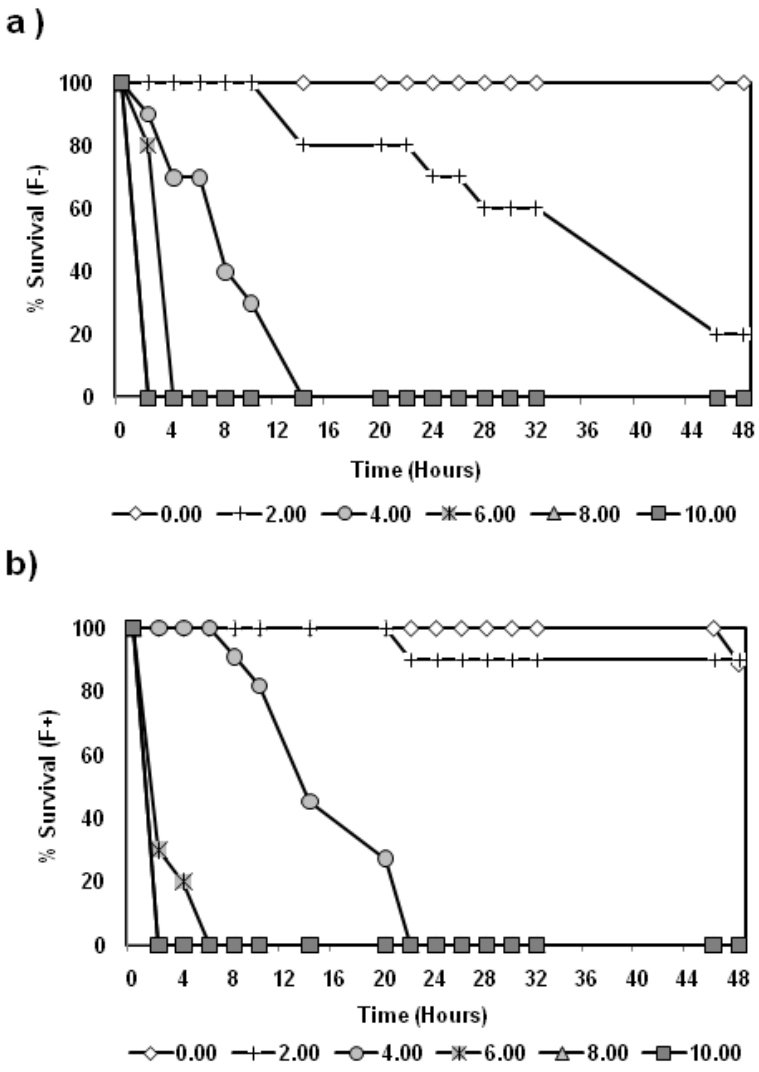

Figure 3.3-1. Percent survival of the *D. pulex* individuals over 48 hours during the first acute exposure to salinity in the absence (F-) (a-upper graph) and presence (F+) (b-lower graph) of the fish-exuded kairomone.

In the second acute toxicity experiment, it was found that daphnid survival was reduced significantly with salinity, the fish-exuded kairomone and time ($p < 0.001$***, Table 3.3-1, Figure 3.3-2). Up to 5.00 g/L salt, there was no significant difference observed on daphnid survival between the absence (F-) and presence (F+) of the fish kairomone, while in the two highest salinity levels (5.00 and 6.50 g/L), daphnid survival was decreased significantly in both conditions (Table 3.3-2, Figure 3.3-2).

The LC_{50} values of salinity in the absence and presence of the fish-exuded kairomone were calculated using Probit analyses (EPA Probit Analysis Program Version 1.5). In the absence of the fish-exuded kairomone, the 24 and 48 hrs LC_{50} values were 4.754 g/L and 3.260 g/L for NaCl. On the other hand, in the presence of the fish-exuded kairomone the 24 and 48 hrs values were 5.040 g/L, and 3.774 g/L for NaCl. When these values are compared, it can be observed that the LC_{50} values are higher in the presence (F+) of the fish-exuded kairomone than in the absence (F-) of the fish-exuded kairomone.

3.3.2 Chronic Toxicity Experiment

Salinity and time both had significant effects on the survival of daphnids either alone or in combination (Table 3.3-1, Figure 3.3-3). In the absence (F-) of the fish-exuded kairomone, daphnid survival rates at salinity concentrations of 0.05, 0.10, and 0.20 g/L were similar to the control group, while in the presence (F+) of the fish-exuded kairomone, daphnid survival rates at salinity concentrations of 0.05, 0.10, 0.20, and 0.40 g/L were similar to the control group (Table 3.3-1). However, survival of the individuals decreased significanty at salinity concentrations of 0.80 and 1.50 g/L. Moreover, in comparison to the absence of fish-exuded kairomone (F-), the positive impact of the fish-exuded kairomone on daphnid survival was observed, but was not significant (Table 3.3-1).

Table 3.3-2. Results of Dunnett's pair-wise comparison (NaCl- F- vs NaCl + F- and NaCl - F+ vs NaCl + F+) applied to the survival data of *D. pulex* over time during two acute exposures to salinity in the absence (F-) and presence (F+) of fish-exuded kairomone.

	First			**Second**	
Comparison to	**0.0 g/L**			**0.00 g/L**	
Treatment	**F-**	**F+**	**Treatment**	**F-**	**F+**
2.0 g/L	.000***↓	0.492	**0.20 g/L**	.994	1.000
4.0 g/L	.000***↓	.000***↓	**0.60 g/L**	.864	1.000
6.0 g/L	.000***↓	.000***↓	**1.20 g/L**	.155	1.000
8.0 g/L	.000***↓	.000***↓	**2.25 g/L**	.919	1.000
10.0 g/L	.000***↓	.000***↓	**3.50 g/L**	.282	.861
			5.00 g/L	.000***↓	.000***↓
			6.50 g/L	.000***↓	.000***↓

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

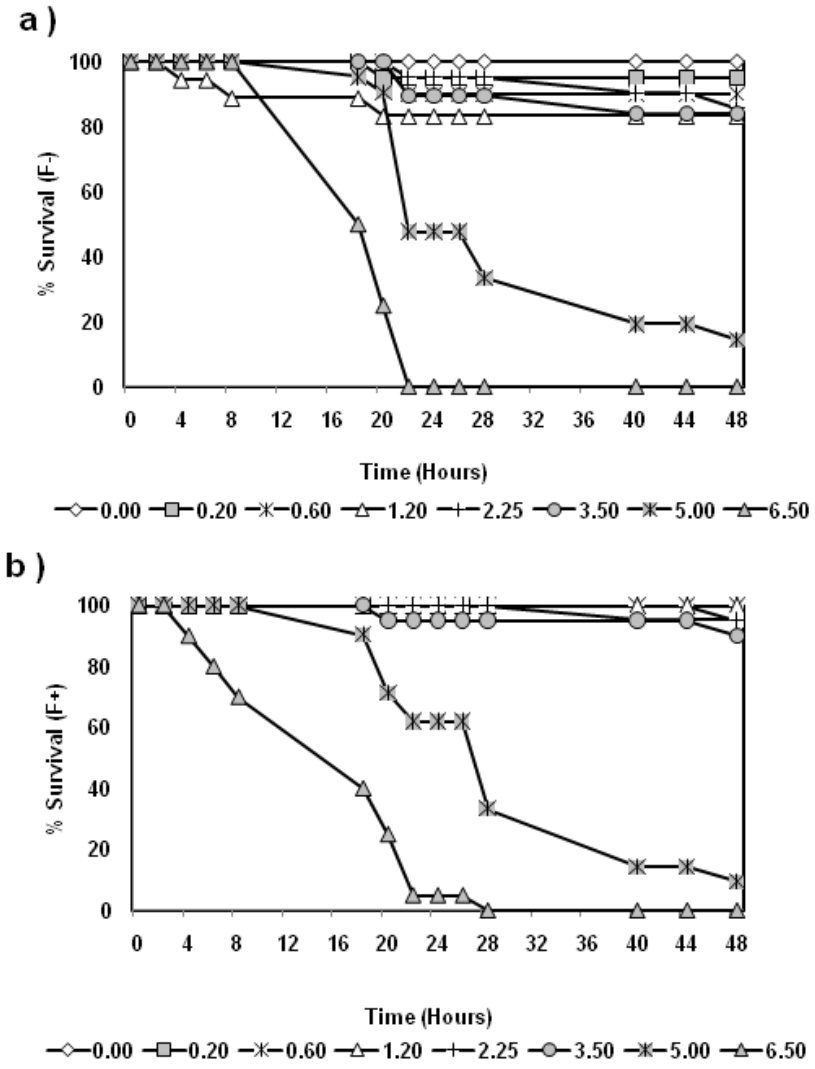

Figure 3.3-2. Percent survival of the *D. pulex* individuals over 48 hours during the second acute exposure to salinity in the absence (F-) (a-upper graph) and presence (F+) (b-lower graph) of the fish-exuded kairomone.

Table 3.3-3. Results of Dunnett's pair-wise comparison (NaCl- F- vs NaCl + F- and NaCl - F+ vs NaCl + F+) applied to survival data of *D. pulex* over time during a chronic exposure to salinity in the absence (F-) and presence (F+) of fish-exuded kairomone.

Treatment	**F-**	**F+**
Comparison to	**0.00 g/L**	
0.05 g/L	.829	1.000
0.10 g/L	.761	1.000
0.20 g/L	.759	.931
0.40 g/L	.051↓	.791
0.80 g/L	.015*↓	.010**↓
1.50 g/L	.003**↓	.002**↓

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

3.3.3 Influence of Salinity on Life History Traits of *Daphnia pulex*

Impacts of salinity on the survival and life history traits of stepwise acclimated *D. pulex* were investigated in a separate chronic toxicity experiment to simulate progressive salt accumulation in a natural ecosystem. It was observed that salinity significantly influenced survival throughout the course of the experiment (Figure 3.3-4, Table 3.3-4), in a decreasing manner (Table 3.3-5). Egg number was also affected significantly by salinity (Table 3.3-4). Table 3.3-5 and Figure 3.3-5 showed that the impact of NaCl decreased the egg number (clutch size) significantly, especially at 0.60, 0.80 and 1.20 g/L NaCl concentrations. Salinity significantly altered body width / body length ratio (Table 3.3-4). Body width to body length ratio significantly decreased with increasing salinity, especially at 0.60, 0.80 and 1.20 g/L salinity concentrations (Table 3.3-5, Figure 3.3-5).

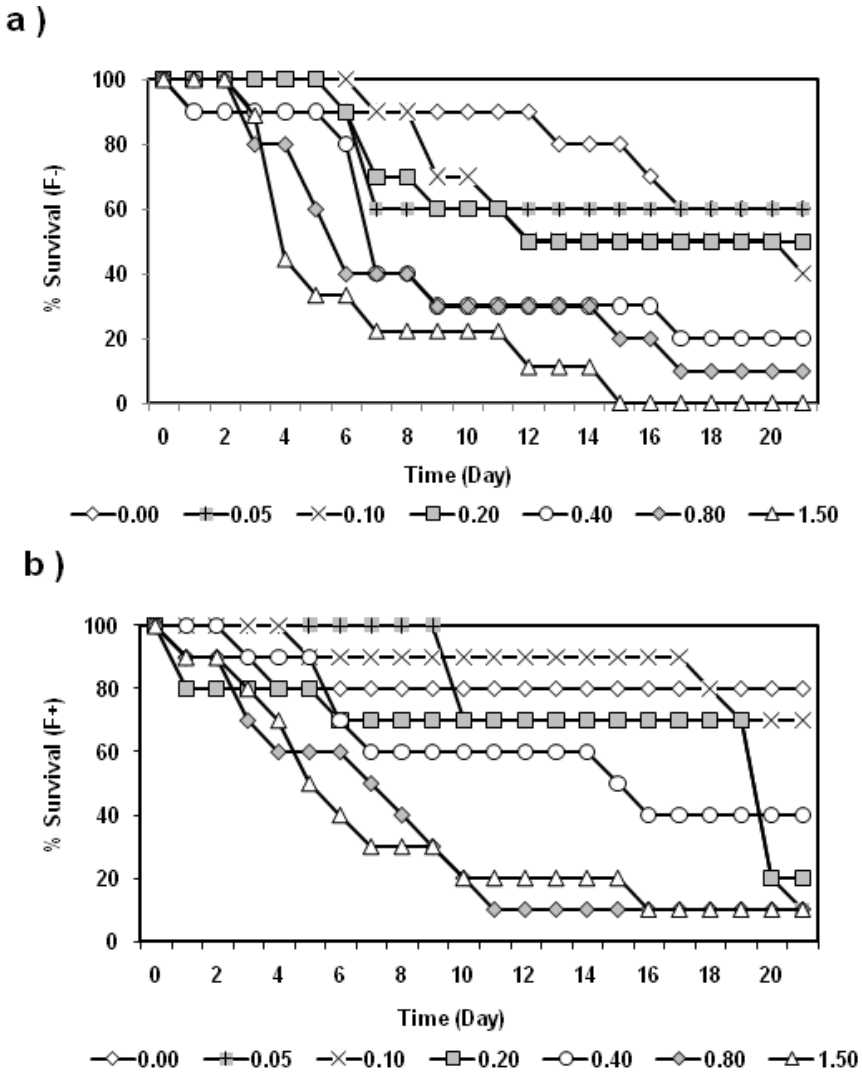

Figure 3.3-3. Percent survival of the *D. pulex* individuals over 21 days chronic exposure to salinity in the absence (F-) (a-upper graph) and presence (F+) (b-lower graph) of the fish-exuded kairomone.

Table 3.3-4. Results of one-way ANOVA applied to the survival, egg number,and body width / body length ratio of acclimated *D. pulex* individuals during a chronic exposure to salinity.

	DF	F	P
Survival	4	4.92	0.002
Egg Number	4	3.53	0.014
Body Width\Body Length	4	4.82	0.003

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

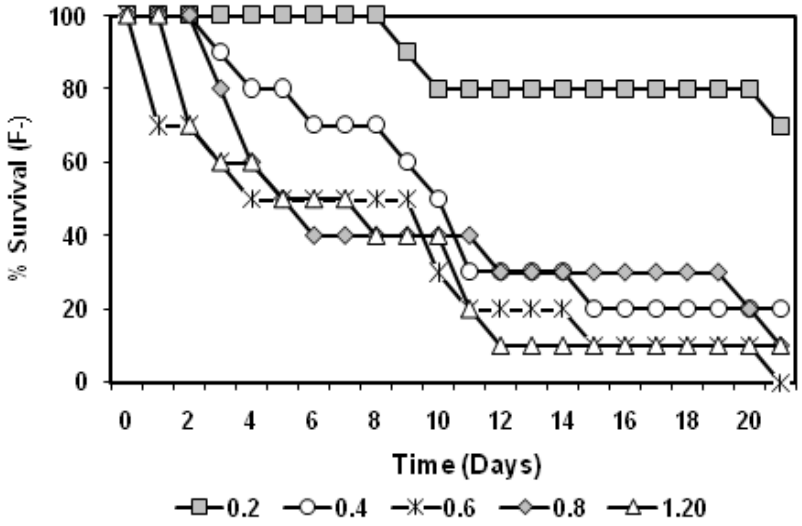

Figure 3.3-4. Percent survival of acclimated *D. pulex* individuals over time during a chronic exposure to salinity.

Table 3.3-5. Results of Dunnett's pair-wise comparison (NaCl- F- vs NaCl + F- and NaCl - F+ vs NaCl + F+) applied to the survival, egg number,and body width / body length ratio of acclimated *D. pulex* individuals during a chronic exposure to salinity.

Comparison to	**0.2 g/L**		
Treatment	**Survival**	**Egg Number**	**Body Width \ Body Length**
0.4 g/L	.041*↓	0.052↓	0.514
0.6 g/L	.002**↓	0.018*↓	0.001***↓
0.8 g/L	.014*↓	0.063↓	0.006**↓
1.2 g/L	.002**↓	0.009**↓	0.000***↓

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

At the end of the experimental processes, survival rates of the non-acclimated and acclimated *Daphnia* individuals were compared and there were no significant differences observed (Table 3.3-6).

Table 3.3-6. Results of one-way ANOVA applied to the survival of non-acclimated and acclimated *D. pulex* individuals during chronic exposures to salinity.

Comparison between Non-acclimated and Acclimated			
Treatment	**DF**	**F**	**P**
0.20	1	1.31	0.267
0.40	1	1.34	0.263
0.80	1	0.01	0.905

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

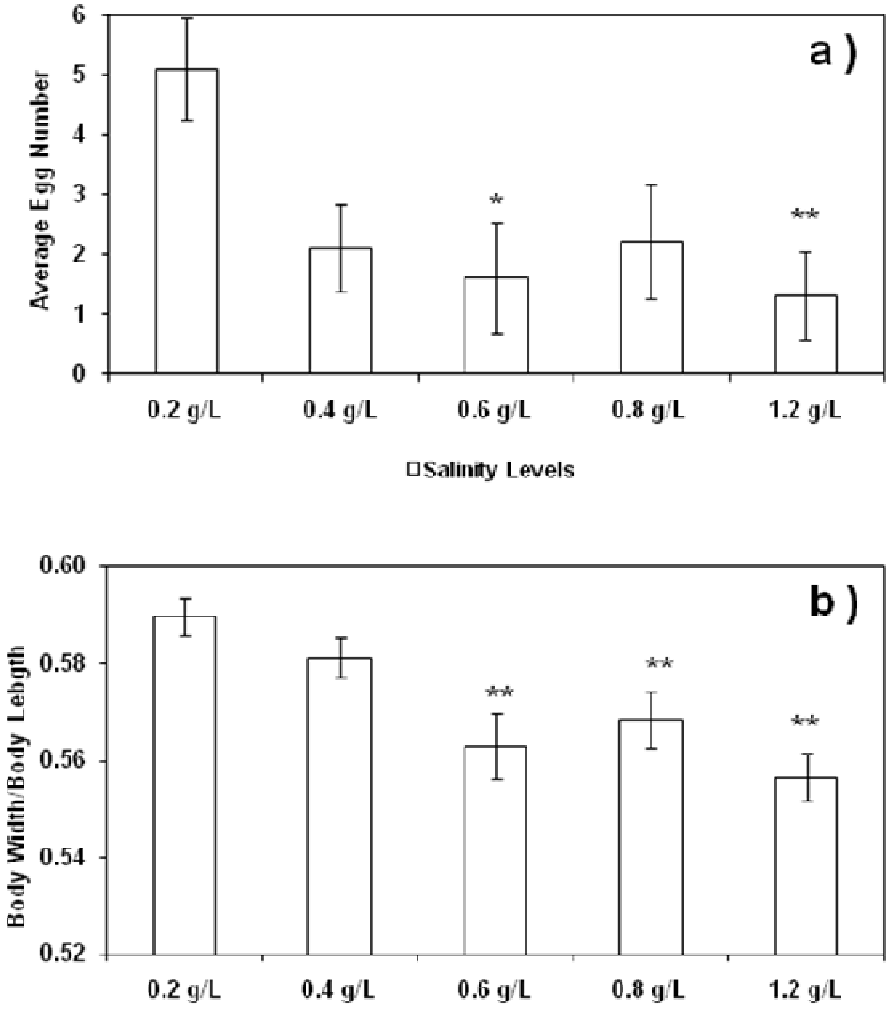

Figure 3.3-5. Average egg number (a) and body width / body length ratio (b) of acclimated *D. pulex* individuals during a chronic exposure to salinity (Mean ± Std Error). Comparison of control and treated samples (NaCl- F- vs NaCl + F- and NaCl - F+ vs NaCl + F+) is by means of the Dunnett's pair-wise comparison test (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

3.3.4 FTIR Spectroscopic Analyses

In this part of this thesis, FTIR spectroscopy was used to measure the alterations in the macromolecules of daphnids jointly exposed to NaCl salinity and the fish-exuded kairomone. For details of ATR-FTIR spectroscopic measurements and band assignment see section 3.1.4.

In the current study, the olefinic =CH band (~3011 cm^{-1}), the CH_2 asymmetric (~2924 cm^{-1}) and symmetric (~2855 cm^{-1}) bands, and the amide I band (~1639 cm^{-1}), which provide information about unsaturated lipids, lipids, and the amide carbonyl stretching vibrations associated with proteins, respectively, were monitored (Giordano *et al*., 2001; Cakmak *et al*., 2003; Severcan *et al*., 2005, Stehfest *et al*., 2005; Akkas *et al*., 2007; Gorgulu *et al*., 2007; Garip *et al*., 2009; Ozek *et al*., 2009).

Figure 3.3-6 presents the alterations in the peak area values of the olefinic, CH_2 asymmetric + CH_2 symmetric, and amide I bands of *D. pulex* individuals chronically exposed to NaCl salinity in the absence and presence of fish-exuded kairomone. These results clearly indicated that with increasing salinity the peak area of the olefinic band (~3011 cm^{-1}) had a general tendency to decrease, which was significant at salinity concentrations ≥ 0.20 g/L. This decrease was significantly counteracted in the presence of the fish-exuded kairomone at salinity concentrations ≥ 0.40 g/L. A similar tendency to decrease with increasing salinity was also observed for the alterations in the summation of the peak area values of the CH_2 asymmetric (CH_2 Asym) (~2924 cm^{-1}) and symmetric (CH_2 Sym) (~2855 cm^{-1}) bands with significance at salinity concentrations ≥ 0.40 g/L. The presence of the fish kairomone again counterbalanced the impact of salinity at concentrations ≥ 0.40 g/L, with significance in the 0.40 g/L (F- vs F+) treatments. The NaCl-induced decrease was also a general tendency for the peak area of the amide I band (~1639 cm^{-1}) of the spectra of daphnids exposed to chronic NaCl exposure in both the control and fish-conditioned treatments.

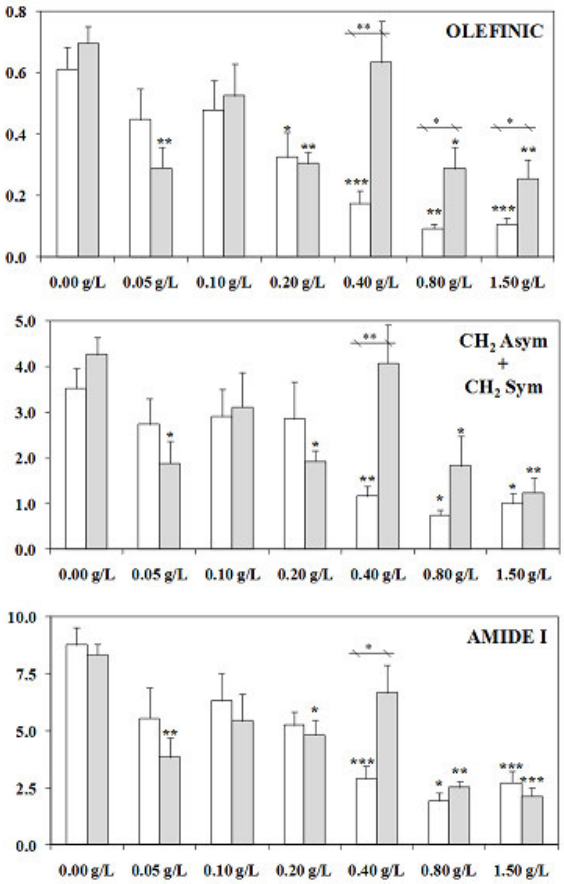

Figure 3.3-6. Peak area values of the olefinic, CH_2 Asymmetric (Asym) + CH_2 Symmetric (Sym), and amide 1 bands determined from the infrared spectra attained from *D. pulex* individuals chronically exposed to NaCl in the absence (open bars) and presence (shaded bars) of fish-exuded kairomone (Mean ± Std Error). Comparison of control and treated samples (NaCl- F- vs NaCl + F- and NaCl - F+ vs NaCl + F+) is by means of the Dunnett's pair-wise comparison test, while comparison of the F- and F+ treatments in identical NaCl treatments is carried out via the Mann-Whitney U test (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

However, the presence of the fish kairomone was again a significant counterbalancing factor at a NaCl concentration of 0.40 g/L. Therefore, in general NaCl salinity leads to decreases in the peak areas of the olefinic, (CH_2 Asym + CH_2 Sym), and amide I bands, which indicate that the contribution of lipids and proteins to the examined *D. pulex* system decreased. In all cases, the decrease induced by salinity stress at concentrations ≥ 0.20 g/L for the olefinic band, and ≥ 0.40 g/L for the (CH_2 Asym + CH_2 Sym) bands and amide I band were significantly reduced by the kairomone in the 0.40 g/L NaCl treatment.

CHAPTER 4

DISCUSSION

4.1 Effects of Pesticide and Fish Predation on *Daphnia pulex*: Organismal and Molecular Approach

It is of great value to develop a better understanding of the impact of pesticides on aquatic ecosystems, which are well known to be highly integrated parts of agricultural areas as providers of water and drainage facilities (Van Wijngaarden *et al*., 2005). Ever since pesticides have been exploited for crop protection, they have inevitably entered aquatic ecosystems and eventually given rise to the field of ecotoxicology (Van Wijngaarden *et al*., 2005; Relyea and Hoverman, 2006). There is critical need to pay attention to the synergistic effects of pesticides and naturals stressors on non-target organisms in order to reveal the underlying mechanisms of pesticide impacts (Relyea and Hoverman, 2006). The current study aimed to investigate the interactive effect of an anthropogenic stressor – namely, the pyrethroid insecticide cypermethrin – combined with a natural stressor – the chemical cue mimicking fish predation – on the survival, reproduction and molecular profile of *Daphnia pulex* by means of bioassays complemented with the advantages of Fourier Transform Infrared (FTIR) spectroscopy.

Synthetic pyrethroids were introduced in the mid-1980s in order to replace the highly toxic organochlorines, organophosphates and carbamates. Cypermethrin is one such registered pyrethroid commonly used to combat pests feeding predominantly on crops of cotton and partially on fruits and vegetables (U.S. EPA, 2008). In Turkey, cypermethrin, one of the top-ranked synthetic pyrethroids, is a

registered active ingredient used in cotton fields (Ministry of Agriculture and Rural Affairs, General Directorate of Protection and Control Statistics, 2004). Nevertheless, exposure to synthetic pyrethroids has great risks even to non-target aquatic species, including zooplankton that share taxonomic similarities with the target organisms (Smith and Stratton, 1986; Day, 1989; Mian and Mulla, 1992).

In nature, zooplankton are exposed to toxicants and natural stressors simultaneously. The assessment of multiple-stressor effects on cladocerans is mostly focused on the interactions of contaminants with food concentration or abiotic factors, such as temperature, heavy metals and pesticides (Folt *et al*., 1999; Barata *et al*., 2006; Kim *et al*., 2008). However, cladocerans – especially large-bodied daphnids – are naturally under the top-down control of fish/invertebrates. Few studies have examined the interactive impact of pesticides and predation pressure – more frequently invertebrate pressure (Barry, 2000; Maul *et al*., 2006; Sakamoto *et al*., 2006). Nevertheless, using fish-exuded kairomone allows us to mimic the natural environments from where test organisms are collected, since they frequently co-exist with fish in relatively high densities as in the case of this study (Beklioglu *et al*., 2003).

In the absence of the fish kairomone, at least 50% of the daphnids survived exposure to 1 µg/L CM even after 48 hrs. However, upon exposure to 5 µg/L CM, about 50% of the daphnids survived only up to 24 hrs. These *D. pulex* survival rates after 24 and 48 hrs are in line with previous studies reporting that the 24- and 48-h EC_{50}/LC_{50} values of *Daphnia magna* exposed to CM are in the range of 0.3-5.0 µg/L (Stephenson, 1982; Day, 1989; EPA ECOTOX Database).

In the presence of the fish kairomone, exposure to 1 µg/L CM further reduced *D. pulex* survival (e.g. after 48 hrs, F+ 1 µg/L treatment 10% survival). The combined effect of CM and fish kairomone was greater than that of either treatment alone and in fact even greater than the sum/product of the effects induced by each stressor alone. Hence, fish kairomone and CM interacted in a synergistic manner and had a

significantly stronger effect. Nonetheless, this synergistic interaction between the pesticide and fish kairomone disappears at 5 μg/L of CM probably because the cypermethrin effect became so strong that it overrode the fish effect.

The extent to which multiple stressors interact with each other varies widely. The occurrence of synergistic interactions is defined as greater impacts of the combined multiple stressors than the sum and/or product of the effects induced by each stressor alone (Folt *et al*., 1999). Even though the fish kairomone alone did not influence the survival of *D. pulex*, the presence of the kairomone exaggerated the negative impact of 1 μg/L CM and resulted in greater mortality. Similar substantial potentiation of toxic effects by fish-based cues has also been observed previously (Maul *et al*., 2006; Beklioglu *et al*., Manuscript to be submitted to Ecotoxicology and Environmental Safety).

There was a concentration-dependent mortality in *D. pulex* at intermediate CM concentrations between 0.04-3.60 μg/L. However, in contrast to the previous high-concentration acute toxicity test, it was observed that the presence of fish kairomone led to increased survival at CM concentrations being ≤ 0.90 μg/L. This implies that at intermediate cypermethrin concentrations, the presence of fish kairomone might act as a positive trigger to resist the existing stress factors. This interaction is one in which multiple stressors are less disturbing than the single dominant stressor alone. Despite the fact that this case is rare, a similar observation was reported by Folt and colleagues (1999). A plausible explanation for the current observation might be the simultaneous occurrence of (i) reduced feeding efficiency and swimming ability of daphnids upon exposure to cypermethrin (Christensen *et al*., 2005) and (ii) greater swimming strength of zooplankton when exposed to greater kairomone signal strength in order to increase effective predation avoidance (van Gool and Ringelberg, 2002). This could explain the increased survival of *D. pulex* at intermediary levels of CM in the presence of the fish chemical cue.

In the re-registration eligibility decision for cypermetrin, it is stated that freshwater invertebrates are substantially more sensitive to cypermethrin toxicity than other aquatic organisms (U.S. EPA, 2008). Hence, the low-concentration chronic toxicity test was performed in order to detect the effect of environmentally relevant cypermethrin concentrations, for which limited research has been carried out (Kim *et al*., 2008), combined with the biotic stress of predator cues, which is an emerging field of interest (Hanazato, 2001; Maul *et al*., 2006; Sakamoto *et al*., 2006). This toxicity test enabled the observation of the interactive impact of fish predation pressure and low-concentrations of CM on *Daphnia pulex* survival and reproduction.

24-72hr old *D. pulex* individuals showed significant mortality at CM concentrations of 11.5 and 15.0 ng/L in both the absence and presence of the fish kairomone. These results are in line with Kim and colleagues (2008), who reported that <24hr old *D. magna* neonates and 7d old *Daphnia* juveniles showed significant mortality at CM concentrations of 2ng/L and 200ng/L, respectively. The presence of the fish-exuded kairomone did not alter the negative effect of the relatively low concentrations of CM on survival.

The production of the first brood (AFR) was delayed at all cypermethrin concentrations, including the lowest (0.5 ng/L). The clutch size decreased significantly in CM exposures of 11.5 and 15.0 ng/L, in part due to decreased survival. These results are in correlation with studies reporting that pesticides – including pyrethroid insecticides such as fenvalerate and cypermethrin – lead to loss of coordination and decreased filtration rates with a consequent reduction in food uptake/assimilation and ultimate delay in onset of reproduction in *Daphnia* species even at sub-lethal concentrations (Gliwicz and Sieniawska, 1986; Christensen *et al*., 2005; Reynaldi *et al*., 2006).

As a result, it is suggested that the fish kairomone-induced life-history shifts may slightly counterbalance the negative effects induced by low-concentrations (0.5-

15.0 ng/L) of cypermethrin, whereas the cue does not affect daphnid survival at these low concentrations. This stress decreasing effect of the fish kairomone can be observed more clearly at intermediate concentrations of CM (0.04-0.90 µg/L), which significantly increased mortality in the absence of the fish-exuded kairomone. However, at higher concentrations of CM (≥ 1.00 µg/L) the presence of the fish kairomone interacts synergistically with CM (F+ 1 µg/L) and results in greater mortality than CM alone (F– 1 µg/L). It is worth noting that this synergistic interaction is lost at 5 µg/L CM, which becomes a single dominant stressor overriding the fish effect.

FTIR spectroscopy is a widely-used absorption technique monitoring the macromolecular pool of ecological samples both sensitively and simultaneously (Cakmak *et al*., 2006; Hirschmugl *et al*., 2006; Palaniappan *et al*., 2008). Chronic exposure to low (11.5-15.0 ng/L) and intermediate (0.04-3.60 µg/L) concentrations of cypermethrin in the absence and presence of the fish kairomone resulted in a decrease in the peak area values of the olefinic band of the infrared spectra of daphnids. The olefinic band (=CH) corresponds to the double bonds in the lipid structure from unsaturated fatty acyl chains (Liu *et al*., 2002; Severcan *et al*., 2005). Many studies have presented that cypermethrin leads to oxidative stress and an induction of defence mechanisms – such as antioxidant enzyme systems – in exposed aquatic species (Meems *et al*., 2004; Li *et al*., 2005). It is well known that unsaturated lipids are more susceptible to lipid peroxidation. Therefore, the decrease in the peak area values of the olefinic band implies a loss of unsaturated lipids due to CM-induced lipid peroxidation (Kinder *et al*., 1997). Notably, these cypermethrin-induced alterations begin even at low concentrations (11.5-15.0 ng/L). Oxidative stress due to such low concentrations of cypermethrin was observed previously as well (Meems *et al*., 2004). Hence, it is crucial to investigate the impact of environmentally relevant cypermethrin concentrations in order to determine early molecular alterations of test organisms. Moreover, the fish-exuded kairomone counterbalanced the decrease in lipid unsaturation (=CH) induced by 0.30 µg/L of CM. This molecular observation is in agreement with the positive

impact of the fish kairomone on daphnid mortality at intermediate CM concentrations. All of these observations may be the molecular background of the organismal responses to cypermethrin and kairomone exposure.

Chronic exposure to intermediate CM concentrations lead to significant decreases in the peak area values of the (CH_2 Asym + CH_2 Sym), amide I, and glycogen bands, implying a decrease in the concentration of these molecules in the daphnid system upon cypermethrin toxicity. It should be underlined here that similar cypermethrin-induced alterations are set off at a CM concentration of 11.5 ng/L, as can be observed from both the ecotoxicological measurements (e.g. survival, clutch size) and the spectral analysis. Therefore, there is decreased contribution of saturated lipids (CH_2 Asym + CH_2 Sym), proteins (amide I) and glycogen (CO-O-C) to the daphnid system after cypermethrin exposure. These results are consistent with previous investigations reporting that the quantity of lipids, proteins and carbohydrates in aquatic species become less pronounced with increased cypermethrin concentrations (Tripathi and Singh, 2004; Begum, 2005; Christensen *et al*., 2005). These reactions may be the consequence of reduced activity of feeding appendages and mandibles in the presence of pesticides and consequent decrease in filtration (Gliwicz and Sieniawska, 1986; Day and Kaushik, 1987). This reduction in the ability to uptake food may result in the depletion of the carbohydrate (e.g. glycogen) reserves, the primary source of energy, to meet the cypermethrin-induced increase in energy demand. The reduction in the protein content may be attributed to (i) the increment in the metabolism of proteins, the reserve energy source for periods of stress, and/or (ii) the impairment of protein synthesis as a consequence of the destruction of cells (Bradbury *et al*., 1987; Barber *et al*., 1990). These molecular alterations may be the underlying metabolic principle of the life-history adaptations in response to stressors observed at the organismal level. The counterbalancing effect of the fish kairomone on the decrease in saturated lipids (CH_2 Asym + CH_2 Sym) induced by 0.30 μg/L of CM might also be the molecular background for the positive impact of the fish kairomone observed at the organism level at intermediate CM concentrations. In

short, fish kairomone-induced alterations at the organismal level were associated with alterations in the unsaturated (=CH) and saturated (CH_2 Asym + CH_2 Sym) lipid bands at the molecular level. Hence, the influence of the fish kairomone may result from inducing an alteration in lipid metabolism. In summary, the changes in the metabolism of daphnids exposed to intermediate and low concentrations of cypermethrin were not only similar to each other but also explanatory to the alterations observed at the organismal level.

As a result, this bioassay-guided spectroscopic approach proves to be promising by providing insight to the complex nature of pesticide-induced alterations at the molecular level, even at concentrations where no adverse effects are observed.

4.2 Molecular Approach to the Effects of Pesticide and Fish Predation on *Daphnia*: An ATR-FTIR Spectroscopic Study

The constraints of insecticides on ecosystems are of major concern because their intention of use is to kill pests in an environment where non-target organisms are also accessible (reviewed by Warren *et al*., 2003; Devine and Furlong, 2007). The use of synthetic pyrethroids, which have both urban and agricultural applications, has been promoted with the intention of replacing organochlorine, organophosphate and carbamate pesticides (Elliot *et al*., 1978). This popularity is the consequence of their high insecticidal activity against target species, relatively lower mammalian and avian toxicity, and rapid biodegradability (Elliot *et al*., 1978; U.S. EPA, 2008). Cypermethrin is one such pyrethroid insecticide used extensively for both indoor and outdoor pest control (U.S. EPA, 2008), in spite of their high toxicity to fish, aquatic invertebrates and other lower aquatic organisms (Smith and Stratton, 1986; Day, 1989; Mian and Mulla, 1992) due to agriculturally and domestically derived insecticide input to various waterbodies in the proximity (Schulz and Liess, 1999; van Wijngaarden *et al*., 2005).

Cypermethrin is acutely and chronically toxic to zooplankton, but the available toxicity data are mostly derived from studies based on single-trophic level testing, which might greatly differ from the 'real-world' where competitive and/or predator-prey interactions are indispensable. Furthermore, cypermethrin is a known neurotoxicant of lipophilic nature with genotoxic effects (Patel *et al*., 2006). However, these effects are generally investigated in fish or mammalian test organisms with limited reports on the molecular basis of cypermethrin toxicity in lower aquatic species (Kim *et al*., 2008). Given the strong need for higher-tier aquatic risk assessment and the lack of information on cypermethrin-induced molecular alterations in lower aquatic invertebrates, the priorities of the current study were two-fold: A novel approach utilizing higher-tier ecotoxicity bioassay-guided ATR-FTIR spectroscopy to better understand the impact of the presence of fish predation pressure – mimicked by predator-exuded info-chemicals – on cypermethrin toxicity to *Daphnia pulex* and ultimately better assess cypermethrin-induced alterations at both organismal and molecular levels.

ATR-FTIR spectroscopy results in complex reflectance spectra (Figure 3.1-5), which enable both qualitative and quantitative analysis. It is worth noting that, infrared spectra give valuable information about the types of bonds and functional groups present in the examined system. Even though infrared data are not appropriate to determine the whole structure of samples, nevertheless they facilitate the characterization of the structure and content of a system. Therefore, the approach of interpretation used in the present study was to seek for consistencies of the trend in alterations in any specific band and correlate such trends between bands of similar origins. Since FTIR studies with dry biological samples are capable of deducing relative quantitative information (Simsek-Ozek *et al*., 2009; Akkas *et al*., Manuscript accepted by Aquatic Sciences), the main point of interest was not to determine the relative changes between macromolecular composition and structure of similar preparations of cypermethrin-treated *Daphnia pulex* individuals in the absence and presence of the fish-exuded kairomone.

All pyrethroids contain a central ester bond and they are primarily metabolized by means of the cleavage of this ester moiety. At this point, it is worth noting that an increase in the peak area values of the C=O ester band, as well as its ratio over the summation of the peak areas of amide I and amide II, was observed in both the absence and presence of the fish-exuded kairomone. It is known that cypermethrin has a main absorption band at 1740 cm^{-1} assigned to carbonyl asymmetric stretch (Segal-Rosenheimer and Dubowski, 2007). The increase in this area of this band with increasing cypermethrin concentration could imply that during the acute toxicity experiment, the cypermethrin molecules were not fully metabolized. These observations support the suggestion that there were only few alterations in the infrared spectra of the daphnids exposed to cypermethrin under acute conditions because of the limited duration of exposure not exceeding 48 hrs.

During the acute toxicity test (1.0-50.0 μg/L) slight molecular alterations were observed despite the fact that the duration of the test was only 48 hrs. The general tendency of the alterations in the peak area values of the bands representing lipids (CH_3 asymmetric, CH_2 asymmetric, and CH_2 symmetric) and proteins (CH_3 symmetric, amide I, and amide II) was to decrease in response to cypermethrin exposure, regardless of the absence or presence of the fish-exuded kairomone. Since the daphnids of the same toxicity experiment could not survive in the face of cypermethrin exposure, these molecular alterations may be the molecular background of the individual level responses. Thus, it can be suggested that cypermethrin leads to reductions in the lipid and protein contents of the examined daphnid system. The molecular response of the *D. pulex* individuals exposed to cypermethrin under chronic conditions was similar. To be precise, the daphnid infrared spectra attained after the chronic toxicity test also showed significant decreases in their lipid (CH_3 asymmetric, CH_2 asymmetric, CH_2 symmetric, ester C=O, and PO_2^- asymmetric) and protein (CH_3 symmetric, amide I, and amide II) content. In addition to these, it was observed that the peak area values of two bands attributed to polysaccharides (1153 cm^{-1} and 1033 cm^{-1}) also showed cypermethrin-induced decreases. These observations strongly suggest that cypermethrin induces

reductions in the lipid, protein and polysaccharide content of daphnids, which may be the molecular explanations of responses observed at the organismal level. A possible explanation of such decreases is that the feeding activity of zooplankton is reduced in the presence of even low concentrations of pesticides (Gliwicz and Sieniawska, 1986; Day and Kaushik, 1987). It is known that type II pyrethroids inhibit the nervous system of insects by affecting nerve cell membranes and thus ion channels (Clark and Brooks, 1989). These action mechanisms of pyrethroids are known to result in loss of coordination (Christensen *et al*., 2005), immobilization (Reynaldi *et al*., 2006), and ultimately reduction in feeding rates (Day and Kaushik, 1987; McWilliam and Baird, 2002) in cladocerans. If such a reduction in the filtering capacity and algae assimilation of *Daphnia* continues, this will result in a major decrease in energy uptake (Christensen *et al*., 2005). Due to this effect on energy uptake, the exploitation of all the reserve energy sources – i.e. lipids, carbohydrates, and proteins – in the body may increase. The observations of the current study not only clarifies the alterations suggested by other studies, but also may be the underlying metabolic principle of the organismal responses of daphnids to stressors.

The HC=CH stretching mode (3013 cm^{-1}) – also known as the olefinic band – in the C-H stretching region (3100-2750 cm^{-1}) can be used as a valuable monitor for the unsaturation in fatty acyl chains (Liu *et al*., 2002; Severcan *et al*., 2005). The peak area of the olefinic band decreased in cypermethrin-treated *D. pulex* in the chronic toxicity test. This indicates that the population of unsaturation in acyl chains of lipid molecules decreased due to cypermethrin exposure. This loss of unsaturation, which is generally due to an increase in lipid peroxidation induced by the presence of free radicals, is supported by several studies suggesting that pesticides induce oxidative stress as a mechanism of their toxic action in animals (Abdollahi *et al*., 2004). Similar cypermethrin-induced oxidative stress and antioxidant activity have been observed for both phytoplankton and zooplankton (Meems *et al*., 2004; Li *et al*., 2005).

It is known from previous studies that a precise lipid-to-protein ratio is another efficient monitor of lipids and proteins, which are crucial macromolecules affecting biological systems (Cakmak *et al*., 2006; Simsek-Ozek *et al*., 2009). In the current study, the lipid-to-protein ratio determined by calculating the ratio of the areas of (CH_2 asymmetric stretch + CH_2 symmetric stretch) to CH_3 symmetric stretch was investigated in detail. In the acute toxicity test, this ratio increased in the absence of the fish-exuded kairomone and decreased in the presence of the fish-exuded kairomone. On the other hand, in the chronic toxicity test the same ratio showed a decrease, regardless of the absence or presence of the fish-exuded kairomone. A possible explanation for the difference between the alterations of this ratio during acute (F-) and chronic (F-) exposures to cypermethrin, where the (CH_2 asymmetric stretch + CH_2 symmetric stretch) to CH_3 symmetric stretch peak area ratio increased in acute conditions and decreased in chronic conditions, may be the actual time of exposure. It is known that direct pesticide effects depend on the duration of exposure (Reynaldi and Liess, 2005). Therefore, the increased lipid-to-protein ratio in the acute experiment may be due to the relatively more pronounced cypermethrin-induced decrease in protein content. However, as the duration of exposure to cypermethrin increases, the utilization of lipid resources is so much greater that the lipid-to-protein ratio alters in a decreasing manner. Barber and colleagues (1990) report that toxicant exposure may reduce assimilation without affecting respiration rate and thus increasing the energy demand for maintenance. Therefore, it could be suggested that acute exposure to cypermethrin may result in an alteration in protein functioning, but as exposure time increases the demand for energy increases dramatically and thus exploitation of lipids overwhelms the exploitation of proteins.

The decrease in the respective lipid-to-protein ratio in the presence of the fish-exuded kairomone, regardless of the time of cypermethrin exposure, indicates that during the presence of the fish-exuded kairomone the decrease in lipids is more marked than the decrease in proteins. This observation was supported by the decreases observed in the lipid-to-protein ratios calculated from the peak areas of

the (i) (CH_2 asymmetric stretch + CH_2 symmetric stretch) to amide I ($p < 0.001$***) and (ii) CH_3 asymmetric stretch to CH_3 symmetric stretch ($p < 0.01$**) at 3.60 µg/L cypermethrin in comparison to the control treatment in the presence of the fish-exuded kairomone. Moreover, it should be highlighted that in all of the lipid-oriented bands the final reduction in the 3.60 µg/L CM-treatment from the control treatment in the absence (F-) of the fish kairomone (CH_3 asymmetric: 71%, CH_2 asymmetric: 81%, CH_2 symmetric: 87%, C=O ester: 95%, and PO_2^- asymmetric: 69%) was higher than in the presence of the fish-exuded kairomone (CH_3 asymmetric: 50%, CH_2 asymmetric: 73%, CH_2 symmetric: 73%, C=O ester: 93%, and PO_2^- asymmetric: 66%). These observations put forward that the decrease induced by cypermethrin is counterbalanced when the fish-exuded kairomone is present and thus results in a lower reduction in lipid content. A similar reversing effect of the fish kairomone was also observed for the olefinic band (=CH) of *Daphnia* individuals exposed to 0.30 µg/L of cypermethrin, as presented in section 3.1. These results, which are all supportive of each other, are likely due to the fact that the influence of the fish kairomone may result from inducing an alteration in lipid metabolism. This suggestion was first proposed in section 3.1. The stress-decreasing impact of the presence of the fish-exuded kairomone was also observed in the survival results (Figure 3.2-1), where daphnid survival rate decreased at cypermethrin concentrations ≥ 0.10 µg/L, while this decrease was reversed in the treatments containing the fish-exuded kairomone at cypermethrin concentrations between 0.10-0.90 µg/L. However, the counterbalancing effect of the fish kairomone observed at the molecular level of the 1.80 and 3.60 µg/L cypermethrin treatments may not be efficient enough as these concentrations are known to be greater than the recognized EC50/LC50 values (Stephenson, 1982; Day, 1989; EPA ECOTOX Database). As a result, all of these observations put forward the idea that the action mechanism of the fish-exuded kairomone – putting forward the molecular aspect of organismal responses – is by means of the lipid metabolism of *Daphnia* exposed to cypermethrin at concentrations between 0.10-0.90 µg/L.

In addition, the wavenumber of the CH_3 asymmetric stretching vibration shifted to higher frequencies in cypermethrin-treated individuals. Shifts in the wavenumber of -CH stretching modes are sensitive monitors of the state of order/disorder of lipids (Casal and Mantsch, 1984). An increase in the frequency of the CH_3 asymmetric stretching vibration indicates a diminished state-of-order of fatty acyl chains due to an increase in the number of gauche conformers (Casal and Mantsch, 1984; Schultz and Naumann, 1991; Kazanci *et al*., 2001). This points out that cypermethrin exposure disorders lipid molecules. It could be possible that cypermethrin-induced lipid disorder is due to its mechanism of action by way of altering ion channel activity (Clark and Brooks, 1989). It is important to appreciate the interrelationship between ion channel kinetics, lipid order/disorder and ultimately physiological disorders (Awayda *et al*., 2004). Thus, the impact of cypermethrin on lipid order may be a mechanism of toxic action resulting in the observations made at the organismal level.

Last but not least; it is also worth pointing out that there were significant alterations in the peak area values of the infrared spectra of *D. pulex* individuals exposed only to the solvent in both the absence (F- SCo) and the presence (F+ SCo) of the fish-exuded kairomone. This effect of the solvent acetone on the molecular profile of daphnids, despite the fact that the solvent control treatment did not influence their survival rate, could imply that solvent concentrations even below concentrations recommended by OECD may have effects at the molecular level. This means that it may not always be possible to conclude that a toxicant does not have any effects from only organismal level responses. What is more, the negative effect of the solvent was more pronounced in the presence of the fish-exuded kairomone, implying that the effect of multiple stressors may not be as foreseen from their singular effects. Such a finding highlighting that natural stressors may bring out the real toxicity of chemicals has also been reported by Beklioglu and colleagues (Manuscript submitted).

The amide I band located at 1642 cm^{-1} provides valuable information on proteins and their secondary structure. The decrease in its peak area, as well as the decrease in the peak area of the amide II band, implies a decreased contribution of proteins *Daphnia* individuals exposed to cypermethrin at chronic levels. The alterations in the intensities of the sub-components of the amide I band, which was analyzed in detail to observe changes in protein structure, presented that the contribution of antiparallel-aggregared β-sheet and random coil structures increased, while the β-sheet structure decreased to the daphnid protein structure. These changes and the shift in the wavenumber of the amide A band could indicate alterations – and likely protein denaturation – in the conformational structure of the protein system of daphnids under chronic exposure to cypermethrin. A possible explanation for the decrease in protein content and denaturation of protein secondary structure is that cypermethrin causes an increase in lipid metabolism, which ultimately leads to the utilization of proteins as an energy source. It is a known fact that extreme lipid and protein degradation for a long period will destroy the metabolism of an organism and this may be a major molecular reason behind the increased mortality of *D. pulex* individuals observed in the toxicity tests.

4.3 Impacts of Salinity and Fish-Exuded Kairomone on the Survival and Molecular Profile of *Daphnia*

Since *Daphnia* is keystone genus of freshwater communities, it is crucial to understand how it will be influenced by both biotic and abiotic stressors in natural environments. Abiotic and biotic stressors, such as temperature, salinity, predation pressure and food availability, have strong control on daphnids population dynamics and life history parameters (Lynch, 1978; DeMott, 1983; Lampert, 1985; Hu and Tessier, 1995; Urabe *et al*., 1997; von Elert, 2002; Ferrao-Filho and Azevedo, 2003). Salinity is one such important abiotic factor, while vertebrate predation pressure is a biotic factor influencing freshwater organisms. The current study was designed to investigate the combined effect of an abiotic stressor – NaCl salinity – and a biotic stressor - vertebrate predation pressure mimicked by fish-

exuded kairomone - on *Daphnia pulex* at both the organismal and molecular levels by means of bioassays complemented with the advantages of Fourier Transform Infrared (FTIR) spectroscopy.

In the first acute toxicity experiment, the aim was to determine the maximum resistance of daphnids to combined effects of high salinity levels (2.00-10.00 g/L) and the fish-exuded kairomone. In the absence of the fish-exuded kairomone (F-), increased salinity significantly decreased the survival of daphnids. Above the 2.00 g/L salinity level, nearly all of the daphnids were dead within 24 hours. However, the decreased survival at 2.00-4.00 g NaCl L^{-1} in the absence of fish-exuded kairomone (F-) was counterbalanced with presence of the fish-exuded kairomone (F+). In other words, the presence of the fish-exuded kairomone enhanced the resistance of daphnids to salinity, where daphnid survival at 2.00 g NaCl L^{-1} was not significantly different from the control group and mortality at 4.00 g NaCl L^{-1} was lower than in the absence of the fish-exuded kairomone. On the other hand, at the salinity levels $\geq$ 6.00 g/L, the daphnids could not survive more than 24 hours, regardless of the presence of the fish-exuded kairomone. This NaCl-induced increased mortality at salinity concentrations over 5.00 g/L was also observed by Sarma *et al*. (2006), who studied *D. pulex* individuals collected from Mexican freshwaters. Thus, the present study found that at high concentrations, the effect of salinity overrid the positive effect of fish.

In the second acute toxicity experiment, similar to the previous one, survival decreased significantly at salinity concentrations $\geq$ 5.00 g/L, regardless of the presence of the fish-exuded kairomone. In the absence of fish-exuded kairomone (F-), for 24 and 48 hr LC_{50} values were lower (4.754 g/L and 3.260 g/L) than the presence of fish exuded kairomone (5.040 g/L, and 3.774 g/L), respectively. These values for *D. pulex* are in line with previous studies reporting that the 24- and 48-h EC_{50}/LC_{50} values of *Daphnia pulex* exposed to NaCl salinity are in the range of 1.38-5.91 g/L (EPA ECOTOX Database). In addition, the positive impact of the fish-exuded kairomone was also observed in the lower concentrations (0.20-3.50

g/L) of this acute toxicity experiment, even though not significant. That is, when the 24 and 48 hr LC_{50} values were compared, it was clearly seen that in the absence of fish-exuded kairomone (F-), daphnids were less resistant to salinity in comparison to the presence of fish-exuded kairomone (F+). Therefore, the *Daphnia* individuals were more successful in coping with salinity stress in the presence of the fish-exuded kairomone, as the LC_{50} values were higher than the respective values in the absence of the fish-exuded kairomone.

According to the results of both acute toxicity experiments, fish-exuded kairomone had a positive impact on daphnid survival up to 5.00 g/L of salt, but at salinity levels greater than 5.00 g/L the observed positive impact of the fish-exuded kairomone was overridden with the toxic effect of salt. In accordance with the simple comparative model, when the effect of multiple stressors are greater than or equal to the effect of the single-worst stressor this combined effect is called synergism, while if the effect of multiple stressors is less than the single dominant stressor, this combination is an antagonistic interaction (Folt *et al*., 1999). Therefore, the interaction of salinity and fish-exuded kairomone is synergistic when NaCl concentration is $\geq$ 5.00 g/L, but antagonistic when NaCl concentration is $<$ 5.00 g/L. Even though antagonism is not widely recorded in the literature, Mason (2002) found that elements like calcium and copper reduced the negative effects of lead, zinc and aluminum.

In order to investigate the antagonistic interaction mentioned above in more detail, a chronic toxicity experiment with a NaCl range of 0.00-1.50 g/L in the absence (F-) and presence (F+) of the fish-exuded kairomone was designed. In this experiment, in the absence of the fish-exuded kairomone, a significant impact of salinity on daphnid survival was observed between 0.40-1.50 g/L NaCl concentrations. This is in line with findings of others, who reported that salinity in the ranges between 0.40-4.00 g/L lead to increased mortality in *D. pulex* and *D. carinata* (Hall and Burns, 2002, Sarma *et al*., 2006). The antagonistic interaction between NaCl and fish-exuded kairomone observed in the acute experiments was

clearly observed during chronic exposure to NaCl. This antagonistic interaction between fish-exuded kairomone and NaCl concentrations was significant in the 0.40 g/L treatment. The stress reducing effect of the kairomone was masked by salinity concentrations of 0.80 and 1.50 g/L and the survival of the daphnids were reduced significantly. Even though the fish-exuded kairomone was expected to present a positive impact on NaCl-induced mortality according to the acute experiments (48 hrs), 0.80 and 1.50 g/L concentrations lead to significant decrease in survival because it is exposed in a chronic manner (21 days) and thus masked the kairomone effect.

The results of the chronic toxicity experiment carried out with acclimated *D. pulex* individuals supported the previous chronic experiment, where chronic exposure to NaCl concentrations between 0.40-1.20 g/L decreased the survival of daphnids significantly. This observation indicates that salinity is a major stress factor on the survival of *Daphnia* individuals, because their survival was still decreased despite the acclimation process, which was employed in order to mimic the salt accumulation in a natural environment.

Under optimum conditions, *Daphnia* individuals use %27 of their energy for assimilation, %68 for reproduction and %5 for growth (Richman, 1958). When they reach optimal body length, they use ¾ of their energy for reproduction (Richman, 1958; Baillieul *et al.*, 1996). However, changes in environmental conditions (including both biotic and abiotic factors such as predation pressure, increased salinity, temperature alteration, lower food availability, etc.) can change the energy budget of daphnids. In this study, it was observed that between the ranges of 0.40-1.20 g/L NaCl, the number of eggs produced declined significantly. Lignot *et al., (*2000) revealed that when daphnids were exposed to salinity stress, they allocated more of their energy to cope with the stress caused by salinity. This may explain the change in their energy balance and thus the observed decrease in energy allocation to egg production. The survival rate and egg number (clutch size) of species are related with population growth (Stearns, 1992, Sarma *et al.*, 2006).

Hence, the observed negative impact of salinity on the survival and egg number (clutch size) of daphnids may result in suppressed growth rates, which will ultimately have a negative effect on the population growth.

Moreover, salinity led to a significant decline in the body width to body length ratio with respect to increasing NaCl levels. These changes in the body shapes could be related with the osmoregulation mechanisms of daphnids, which aim to balance the higher levels of salt in the environment and consequently lose water. A similar observation was made by Teschner (1995), who reported that the size at first reproduction was decreased under salinity stress. It is also reported that salt may limit individuals' growth rates, even though it may not reduce lifespan. For instance, *Daphnia carinata* and *D. magna* were observed to grow more slowly when they were transferred to brackish environments (Arnér and Koivisto, 1993; Teschner, 1995; Hall and Burns, 2002). Therefore, changes in the life-history traits are related to the costs of living in conditions of salinity stress. Such changes may be the consequences of the high costs of living under salinity stress and thus the potential ability of daphnids to adapt may be overshadowed (Grzesiuk and Mikulski, 2006).

In the current part of the thesis, Fourier Transform Infrared (FTIR) Spectroscopy was used as a molecular tool complementing the organismal data obtained from the chronic salinity ecotoxicity tests. The olefinic band arises from the =CH double bonds in the fatty acyl chains of unsaturated lipids (Liu *et al*., 2002; Severcan *et al*., 2005), while the CH_2 asymmetric and CH_2 symmetric stretching vibrations mainly originate from lipid acyl chains (Akkas *et al*., 2007; Garip *et al*., 2009). Chronic exposure to NaCl salinity (0.05-1.50 g/L) as a stressor both in the absence and presence of the fish kairomone resulted in a decrease in the peak area values of the olefinic band and the summation of the peak area values of the CH_2 asymmetric and symmetric bands of the infrared spectra of *D. pulex*. The significant decrease in the peak area of the olefinic band with exposure to salinity concentrations $\geq$ 0.20 g/L indicates a decrease in the population of unsaturation in acyl chains of lipid

molecules. This loss of unsaturation may be due to NaCl-induced lipid peroxidation. Likewise, the significant decrease in the (CH_2 Asym + CH_2 Sym) bands as a result of salinity exposure to concentrations ≥ 0.40 g/L suggests a reduction in the amount of lipids and possibly an alteration in the composition of acyl chains (Cakmak *et al*., 2006). Cailleaud and colleagues (2007) have presented that salinity stress could interfere with oxidative stress responses and modify antioxidative activities in *Eurytemora affinis*, a calanoid copepod. Such a mechanism could be the basis of the alterations in the amounts of unsaturated and saturated lipids, as lipids undergo peroxidation and are major targets of free radical attack. Moreover, the NaCl-induced increase in lipid peroxidation was significantly counterbalanced in the presence of the fish-exuded kairomone at salinity concentrations ≥ 0.40 g/L. Likewise, the NaCl-induced decrease in lipid content was reversed towards control values in the presence of the fish-exuded kairomone at salinity concentrations 0.40 g/L ($p < 0.01$**), and ≥ 0.80 g/L ($p > 0.05$). These molecular observations are in agreement with the daphnid survival data, where a similar improvement in survival due to the presence of the kairomone was observed for salinity treatments at concentrations ≥ 0.40 g/L. Therefore, these alterations in the saturated and unsaturated lipids may be the molecular background of the organismal responses to NaCl. What is more, it may be by means of the lipid metabolism that the fish-exuded kairomone exerts its positive impact on daphnids. We had similar observations for daphnids exposed to a pesticide and fish-exuded kairomone simultaneously, as well (Akkas, *et al*., submitted).

The alterations in the peak area values of the investigated protein absorption signal - namely the amide I band - had a tendency to decrease with ascending NaCl concentration. Therefore, there was a reduction in the concentration of proteins in *D. pulex* individuals exposed to NaCl, especially significant at concentrations ≥ 0.40 g/L, and this decrease was reversed by the fish-exuded kairomone at 0.40 g NaCl L^{-1} ($p < 0.05$*). It has been reported that variations in ionic strength not only alter the conformation of proteins and DNA, but also the interactions between proteins and DNA (Favre and Rudin, 1996). Hence, the decline in protein

concentration observed in the current study may be due to salinity-induced effects on the structural properties of proteins.

It should be emphasized that the organismal observations made during this chronic exposure of *D. pulex* to NaCl are explained by the molecular observations attained from infrared spectral analysis. To be exact, the NaCl-induced decreases in survival at salinity concentrations ≥ 0.40 g/L correspond to decreases in the lipid and protein content of the *Daphnia* system at the same salinity concentrations. The decreases in the lipid and protein content may be the consequence of the reduction in the ability of daphnids to filter-feed with exposure to higher levels of salinity (Soucek, 2007). This decreased level of energy input to the metabolism of daphnids is the likely reasoning behind decreased growth and reproduction rates at elevated salinities (Arnér and Koivisto 1993), explaining the decreased fecundity and body size observed in the current study. In other words, the spectral data may represent the molecular response initiating the reaction of an organism to its environment.

These correlations between ecotoxicological and spectral data are further strengthened by the counterbalancing effect of the fish-exuded kairomone observed for both organismal and molecular data. It is worth emphasizing that especially for the 0.40 g NaCl L^{-1} treatment, where there was a significant decrease in survival in the absence of the fish-exuded kairomone but the percent survival was closer to the control when the kairomone was present, similar reversing effects of the kairomone was also observed in the survival and spectral data, where the NaCl-induced decrease in lipid and protein concentrations were counterbalanced by the fish-exuded kairomone. In short, the fish kairomone-induced alterations at the organismal level can be correlated with the alterations in the lipid and protein bands at the molecular level. Moreover, it is suggested that the influence of the fish-exuded kairomone may be strongly linked with the lipid metabolism of daphnids, as there is a stronger correlation between the presence of the kairomone and the lipid bands, especially unsaturated lipids (=CH). These results are consistent with previous investigations reporting the simultaneous impact of

cypermethrin and the fish-exuded kairomone on *D. pulex* individuals (please refer to sections 3.1and 3.2). In summary, the molecular data had robust explanatory power to the alterations in the organismal observations.

As a result, this novel approach of complementing toxicity tests with infrared spectroscopy proves to be promising, as it provides molecular insight to the organismal alterations.

CHAPTER 5

CONCLUSION

Summary – The ecotoxicity tests and spectral analyses clearly showed that cypermethrin has adverse effects on the survival, reproduction and metabolic profiles of *D. pulex* and the fish-exuded kairomone is a major driving factor affecting the severity of the impact of cypermethrin in a concentration-dependent manner. It was observed that the fish kairomone compensated for the alterations in *D. pulex* life-history traits and survival inflicted by low and intermediary concentrations of cypermethrin, respectively. In contrast, the fish chemical cue actually had a synergistic impact on high concentrations of cypermethrin. The conceptual representation of the impact of fish-exuded kairomone on cypermethrin-induced *D. pulex* mortality during the three different experiments of this study can be seen in Figure 5-1. Accordingly, the presence of fish-exuded kairomone decreases the stress induced by cypermethrin below a breaking point (i.e. when fish kairomone is present, the CM concentration resulting in 40% mortality is slightly more), while it increases the stress induced by cypermethrin above that same threshold (i.e. when fish kairomone is present, the CM concentration resulting in 80% mortality is much less). Hence, based on the concentrations used in the current study, the fish kairomone seems to increase survival (i.e. negative mortality) below the breaking point. However, as the CM concentration increases, the fish kairomone exaggerates daphnid mortality up to a level after which its effect is sharply lost. Such different types of interactions between multiple stressors may be based on a system of hysteresis with a number of states.

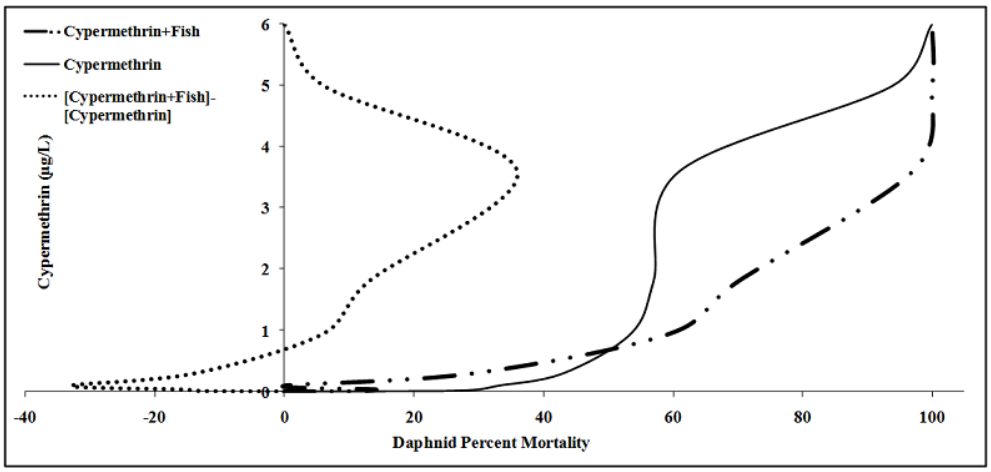

Figure 5-1. A conceptual representation of the impact of fish-exuded kairomone on cypermethrin-induced *D. pulex* mortality at low, intermediate and high concentrations of cypermethrin.

Another crucial observation of the current study is the counterbalancing effect of the presence of the fish-exuded kairomone, which mimics the presence of fish predation pressure. It was observed that the fish-exuded kairomone did not alter the slight impact of at low concentrations of cypermethrin (0.5-15.0 ng/L). However, at intermediate cypermethrin concentrations (0.04-3.60 µg/L), the fish-exuded kairomone may exert a stimulatory effect on daphnid survival and reverse the molecular alterations induced by cypermethrin. Nevertheless, this positive effect of the fish kairomone may be turned into a exaggerating effect at high cypermethrin concentrations (1.0-5.0 µg/L). Therefore, the available evidence suggests that the interactive relationship between exposure to a toxicant and fish-exuded kairomone may be adjusted to a threshold effect level, as in the case of the hormesis phenomenon. *Hormesis* is defined as an adaptive response to low levels of stress that results in improved fitness for a finite period (Calabrese and Baldwin, 2002). The occurrence of hormesis is concidered to be an evolutionary adaptation facilitating the maintenance of fitness in a changing environment (Forbes, 2000). Hormesis, a phenomenon in which the concentration-response relationship is non-monotonic and is thus U-shaped, is suggested to be a rule rather than an exception,

because it is independent of phyla, chemical class of toxicant, and biological endpoint measured within toxicity test (Calabrese and Baldwin, 1999; Zala and Penn, 2004). As a result, the current study could present an example to better understand hormesis in the context of:

(i) ecological risk assessment, which is more concerned with effects at the population level and higher, and has received less attention in comparison to human health risk assessment, which is more concerned with individual level responses; and

(ii) biological (i.e. non-chemical) stressors, which are important in shaping ecosystems, but have not received as much attention as hormesis in chemical stressors (van der Schalie and Gentile, 2000).

Nevertheless, the "intermediate disturbance hypothesis" is an ecological phenomenon that has – in theory – intriguing similarities with the hormetic pattern. This points out the importance of exploring the relevance of hormesis to both ecological risk assessment and biological stressors (van der Schalie and Gentile, 2000).

To the best of our knowledge, this study is one of the first that combined the effects of salinity and fish-exuded kairomone on the survival of *Daphnia pulex*. The results solidify the fact that *Daphnia* were very sensitive to changes in environmental conditions. Although, there is a positive (antagonistic) impact of the fish-exuded kairomone on the daphnid survival, it must be taken into account that in our changing world with respect to global warming, increasing salinity stress may have dreadful results on the food chain of lakes because it reduces the survival of *Daphnia,* which is a keystone genus as a an important grazer on the phytoplankton and a food source for fish and invertebrates.

It is crucial to investigate the combined impact of environmentally relevant concentrations of toxicants and the fish-exuded kairomone on *D. pulex* molecular profile in order to determine early molecular alterations of test organisms. It can be safely said that both cypermethrin and salinity lead to decreased contributions of

lipid and proteins; and possibly altered their conformations in the investigated daphnid systems. Moreover, it is suggested that the action mechanism of the fish-exuded kairomone is via the lipid metabolism of *Daphnia*. The results of the current thesis reveal that the organismal and molecular approaches were clearly consistent with each other. The changes in the daphnid molecular profile based on FTIR spectral data were in close correlation with the changes observed at the organismal level via standard ecotoxicology tools (i.e. current risk assessment tools). For this reason it is suggested that FTIR spectroscopy should be further recognized as a rapid and functional tool utilized more frequently in the future to better understand ecological phenomenon. The application of ATR-FTIR spectroscopy in an ecotoxicological context for comparing the molecular modifications in daphnids exposed to a(n) abiotic and biotic stressor simultaneously, and observing an hormetic effect, which has been reported only once recently (Mecozzi *et al*., 2007), is an important novelty of the current thesis.

In *conclusion*; this thesis puts forward that:

1) *D. pulex* are adversely affected by even low concentrations of cypermethrin and salinity, effects of which are observable at both molecular and organismal levels of biological organization with close correlation,

2) Fish-exuded kairomone is a major driving factor affecting toxicant severity of both cypermethrin and salinity in a concentration-dependent manner, such that;
- The kairomone interacted antagonistically with cypermethrin or salinity below a threshold and synergistically above,

3) Both cypermethrin and salinity lead to decreased contributions of lipid and proteins to the investigated daphnid systems, implying increased energy demands. Furthermore, it is likely that the fish-exuded kairomone targets the lipid metabolism of *Daphnia*,

4) It is possible to increase the environmental realism of aquatic risk assessment by simultaneously merging standard ecotoxicity tests with the impact of predation pressure and monitoring both individual level responses and metabolic profiles of the test organisms.

CHAPTER 6

FUTURE STUDIES

This work will shed light to future studies. With the consideration of the following improvements, the contributions of such ecotoxicological studies to risk assessment can be taken a step forward from this thesis:

- Using a single clone,
- Employing a flow-through experimental setup ,
- Measuring the level of oxygen in the experimental media,
- Verifying the actual concentration of pesticides in the experimental media,
- Taking into consideration the time of death of test organisms into statistical analysis,
- Following the uptake of fluorescence labelled-cypermethrin by *Daphnia* in the absence and presence of the fish-exuded kairomone.

REFERENCES

Abdollahi, M., Ranjbar, A., Shadnia, S., Nikfar, S., and Rezaie, A. (2004) Pesticides and oxidative stress: a review. Med. Sci. Monit., 10(6): RA141-147.

Adams, S.M., Greeley, M.S.Jr., and Ryon, M.G. (2000) Evaluating effects of contaminants on fish health at multiple levels of biological organization: Extrapolating from lower to higher levels. Hum. Ecol. Risk Assess., 6(1): 15-27.

Ahas, R. (1999) Long-term phyto-, ornitho- and ichthyophenological time-series analyses in Estonia. Int. J. Biometeorol., 42(3): 119-123.

Akkas, S.B., Severcan, M., Yilmaz, O., and Severcan, F. (2007) Effects of lipoic acid supplementation on rat brain tissue: an FTIR and neural network study. Food Chem., 105: 1281-1288.

Arnér, M., and Koivisto S. (1993) Effects of salinity on metabolism and life history characteristics of *Daphnia magna*. Hydrobiologia, 259: 69-77.

Awayda, M.S., Shao, W., Guo, F., Zeidel, M., and Hill, W.G. (2004) ENaC-membrane interactions: regulation of channel activity by membrane order. J. Gen. Physiol., 123: 709-727.

Baer, K.N., and Gouldence, C.E. (1998) Evaluation of high-hardness COMBO medium and frozen algae for *Daphnia magna*. Ecotoxicol. Environ. Saf., 39(3): 201-206.

Baillieul, M., Selens, M., and Blust, R. (1996) Scope for growth and fitness of *Daphnia magna* in salinity-stressed conditions. Funct. Ecol., 10(2): 227-233.

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

Barber, I., Baird, D.J., and Calow, P. (1990) Clonal variation in general responses of *Daphnia magna* Straus to toxic stress. II. Physiological effects. Funct. Ecol., 4: 409-414.

Barry, M.J., and Davies, W. (2004) Effects of invertebrate predators and a pesticide on temporary pond microcosm sused for aquatic toxicity testing. Environ. Pollut. 131: 25-34.

Barry, M.J. (2000) Effects of endosulfan on *Chaoborus*-induced life-history shifts and morphological defenses in *Daphnia pulex*. J. Plankton Res., 22(9): 1705-1718.

Baudo, R. (1987) Ecotoxicological testing with *Daphnia*. Peters, R.H. and De Bernardi, R. (eds)., '*Daphnia*', Mem. Ist. Ital. Idrobiol., 45: 461-482.

Beardall, J., Berman, T., Heraud, P., Kadiri, M.O., Light, B.R., Patterson, G., Roberts, S., Sulzberger, B., Sahan, E., Uehlinger, U. and Wood, B. (2001) A comparison of methods for detection of phosphate limitation in microalgae. Aquat. Sci., 63: 107-121.

Begum, G. (2005) In vivo biochemical changes in liver and gill of *Clarias batrachus* during cypermethrin exposure and following cessation of exposure. Pestic. Biochem. Phys., 82: 185-196.

Beklioglu, M., Cetin, A.G., Zorlu, P., and Ay-Zog, D. (2006) Role of planktonic bacteria in biodegradation of fish-exuded kairomone in laboratory bioassays of diel vertical migration. Arch. Hydrobiol., 165: 89-104.

Beklioglu, M., Gozen, A.G., Yıldırım, F., Zorlu, P., and Onde, S. (2008) Impact of food concentration on diel vertical migration behaviour of *Daphnia pulex* under fish predation risk. Hydrobiologia, 614(1): 321-327.

Beklioglu, M., Ince, O., and Tuzun, I. (2003) Restoration of eutrophic Lake Eymir, Turkey, by biomanipulation undertaken following a major external nutrient control I. Hydrobiologia, 489: 93-105.

Beklioglu, M., and Ozen, A. (2007) Ülkemiz sığ göllerinde kuraklık etkisi ve ekolojik tepkiler. Uluslararası Küresel İklim Değişikliği ve Çevresel Etkiler Konferansı Bildiriler kitabı, pp. 299-306.

Bezirci, G. (2008) Impacts of multistressors on the survival and life history traits of *Daphnia pulex*. M.Sc. Thesis, METU, Ankara, 125 p.

Blaise C, and Férard J-F. (2005) Small-scale freshwater toxicity investigations. Volume. 1: Toxicity test methods. Springer Science & Business. 551 p.

Boersma, M., Spaak, P., and De Meester, L. (1998) Predator-mediated plasticity in morphology, life-history, and behavior of *Daphnia*: The uncoupling of responses. Am. Nat., 152: 237-248.

Bollens, S.M., and Frost, B.W. (1991) Diel vertical migration in zooplankton: rapid individual response to predators. J. Plankton Res., 13: 1359-1365.

Boudou, A., and Ribeyre, F. (1997) Aquatic ecotoxicology: From the ecosystem to the cellular and molecular levels. Environ. Health Persp., 105: 21-35.

Boxall, A.B.A., Brown, C.D., and Barrett, K.L. (2002) Higher-tier laboratory methods for assessing the aquatic toxicity of pesticides. Pest Manag. Sci., 58(7): 637-648.

Bozkurt, O., Bilgin, M.D., and Severcan, F. (2007) The effect of diabetes mellitus on rat skeletal extensor digitorum longus muscle tissue: An FTIR study. Spectroscopy., 21:151-160.

Bradbury, S.P. and Coats, J.R. (1989) Comparative toxicology of the pyrethroid insecticides. Rev. Environ. Contam. Toxicol., 108: 133-177.

Bradbury, S.P., Mckim, J.R., and Coats, J.R. (1987) Physiological responses of rainbow trout *Salmo gairdneri* to acute fenvalerate intoxication. Pestic. Biochem. Physiol., 27: 275-285.

Brooks, J. L. and Dodson, S.I. (1965) Predation, body size, and composition of plankton. Science, 13: 28-35.

Brown, W.L. Jr., Eisner, T., and Whittaker, R.H. (1970) Allomones and kairomones: transspecific chemical messengers. BioScience, 20: 21-22.

Buckler, D.R., Mayer, F.L., Ellersieck, M.R., and Asfaw, A. (2005) Acute toxicity value extrapolation with fish and aquatic invertebrates. Arch. Environ. Contam. Toxicol., 49(4): 546-558.

Buckler, D.R., and Tillitt, D.E. (1996) Biochemical and physiological indicators of contaminant stress in aquatic organisms of large river systems, in: Schoettger, R.A. (Ed.), Problems of Aquatic Toxicology, Biotesting and Water Quality Management. U.S. EPA, EPA/600/R-96/090, North Carolina, pp. 162-174.

Burak, S., Duranyıldız, I., and Yetiş, U. (1997) Ulusal Çevre Eylem Planı: Su Kaynaklarının Yönetimi. DPT, Ankara.

Burks, R.L., Jeppesen, E., and Lodge, D.M. (2000) Macrophyte and fish chemicals suppress *Daphnia* growth and alter life-history traits. Chemical communication in aquatic systems. Oikos, 88(1): 139-147.

Burns, C.W. (1968) The relationship between body size of filter-feeding *Cladocera* and the maximum size of particles ingested. Limnol. Oceanogr., 13: 675-678.

Cailleaud, K., Maillet, G., Budzinski, H., Souissi, S., and Forget-Leray, J. (2007) Effects of salinity and temperature on the expression of enzymatic biomarkers in *Eurytemora affinis* (Calanoida, Copepoda). Comp. Biochem. Physiol. A 147: 841-849.

Cairns Jr., J. (1983) Are single species toxicity tests alone adequate for estimating environmental hazard? Hydrobiologia, 100: 47-57.

Cakmak, G., Togan, I., and Severcan, F. (2006) 17β-Estradiol induced compositional, structural and functional changes in rainbow trout liver, revealed by FT-IR spectroscopy: A comparative study with nonylphenol. Aquat. Toxicol., 77: 53-63.

Cakmak, G., Togan, I., Uguz, C., and Severcan, F. (2003) FT-IR spectroscopic analysis of rainbow trout liver exposed to nonylphenol. Appl. Spectrosc., 57: 835-841.

Calabrese, E.J., and Baldwin, L.A. (1999) Reevaluation of the fundamental dose: Response relationship. BioScience, 49(9): 725-732.

Calabrese, E.J., and Baldwin, L.A. (2002) Defining hormesis. Human Exp. Toxicol., 21: 91-97.

Calow, P. (1998) Ecological risk assessment: Risk for what? How do we decide? Ecotox. Environ. Saf., 40: 15-18.

Campbell, I.D. and Dwek, R.A. (1984) Biological spectroscopy, Benjamin/ Cummings Publishing Company, USA, 1-60.

Carpenter, S.R., Kraft, C.E., Wright, R., He, X., Soranno, P.A., and Hodgson, J.R. (1992) Resilience and resistance of a lake phosphorus cycle before and after food web manipulation. Am. Nat., 140: 781-798.

Casida, J.E., and Quistad, G.B. (1998) Golden age of insecticide research: Past, present, or future? Annu. Rev. Entomol. 43: 1-16.

Charmantier, G., and Charmantier-Daures, M. (2001) Ontogeny of osmoregulation in crustaceans: the embryonic phase. Am. Zool., 41: 1078-1089.

Christensen, B.T., Lauridsen, T.L., Ravn, H.W., and Bayley, M. (2005) A comparison of feeding efficiency and swimming ability of *Daphnia magna* exposed to cypermethrin. Aquat. Toxicol., 73: 210-220.

Clark, J.M., and Brooks, M.W. (1989) Neurotoxicity of pyrethroids: Single or multiple mechanisms of action? Environ Toxicol. Chem., 8: 361-372.

Cold, A., and Forbes, V.E. (2004) Consequences of a short pulse of pesticide exposure for survival and reproduction of *Gammarus pulex*. Aquat. Toxicol., 67: 287-299.

Columé, A., Diewok, J., and Lendl, B. (2004) Assessment of FTIR spectrometry for pesticide screening of aqueous samples. Intern. J. Environ. Anal. Chem. 84(11): 835-844.

Connell, D.W. and Miller, G.J. (1984) The chemistry and ecotoxicology of pollution. Wiley, New York.

Cowgill, U.M., and Milazzo, D.P. (1990) The sensitivity of two cladocerans to water quality variables: salinity and hardness. Arch. Hydrobiol., 120(2): 185-196.

Dağ, S., Aykaç, T., Gündüz, A., Kantarcı, M., and Şişman, N. (2000) Türkiyede tarım ilaçları endüstrisi ve geleceği. Türkiye Ziraat Mühendisliği V. Teknik Kongresi, 2, 933, Ankara.

Datta, M., and Kaviraj, A. (2003) Acute toxicity of the synthetic pyrethroid deltamethrin to freshwater catfish *Clarias gariepinus*. Bull. Environ. Contam.Toxicol., 70: 296-299.

David, M., Mushigeri, S.B., Shivakumar, R., and Philip, G.H. (2004) Response of *Cyprinus carpio* (Linn) to sublethal concentration of cypermethrin: alterations in protein metabolic profiles. Chemosphere, 56: 347352.

Day, K., and Kaushik, N.K. (1987) Short-term exposure of zooplankton to the synthetic pyrethroid Fenvalerate and its effects on rates of filtration and assimilation of the algae *Chlamydomonas reinhardii*. Arch. Environ. Con. Tox., 16: 423-432.

Day, K.E. (1989) Acute, chronic and sublethal effects of synthetic pyretroids on freshwater zooplankton. Environ. Toxicol. Chem., 8: 411-416.

De Coen, W.M., and Janssen, C.R. (1998) The use of biomarkers in *Daphnia magna* toxicity testing. I. The digestive physiology of daphnids exposed to toxic stress. Hydrobiologia, 367: 199-209.

De Meester, L., and Weider, L. (1999) Depth-selection behavior, fish kairomones, and the life histories of *Daphnia hyalina* x *galeata* hybrid clones. Limnol. Oceanogr., 44: 1248-1258.

Dean A.P., Martin, M.C., and Sigee, D.C. (2007) Resolution of codominant phytoplankton species in a eutrophic lake using synchrotron-based Fourier transform infrared spectroscopy. Phycologia, 46(2): 151-159.

Dean, A.P., Estrada, B., Nicholson, J.M., and Sigee, D.C. (2008) Molecular response of *Anabaena flos-aquae* to differing concentrations of phosphorus: A combined Fourier transform infrared and X-ray microanalytical study. Phycological Research., 56: 193-201.

DeMott, W.R. (1983) Seasonal succession in natural *Daphnia* assemblage. Ecol. Monogr., 53: 321-340.

Demoute, J.P. (1989) A brief review of the environmental fate and metabolism of pyrethroids. Pestic. Sci., 27: 375-385.

Devine, G.J., and Furlong, M.J. (2007) Insecticide use: Contexts and ecological consequences. Agr. Human Values., 24: 281-306.

Diem, M. (1993) Introduction to modern vibrational spectroscopy, John Wiley & Sons, USA.

Dodson, S.I. (1974) Adaptive change in plankton morphology in response to size-selection predation: a new hypothesis for cyclomorphosis. Limnol. Oceanogr., 19: 721-729.

Dodson, S.I. (1988) The ecological role of chemical stimuli for the zooplankton: Predator-avoidance behaviour in *Daphnia*. Limnol. Oceanogr., 33: 1431-1439.

Dodson, S.I., Frey, D.G. (2001) Cladocera and other branchiopoda. In: Thorp JH, Covich AP (eds) Ecology and classification of North American Freshwater invertebrates. Academic Press, London, pp 850-914.

Dodson, S.I., Merritt, C.M., Shannahan, J.P., and Shults, C.M. (1999) Low exposure concentrations of atrazine increase male production in *Daphnia pulicaria*. Environ. Toxicol. Chem., 18(7): 1568-1573.

Doksaeter, A., and Vijverberg, J. (2001) The effects of food and temperature regimes on life-history responses to fish kairomones in *Daphnia hyalina* x *galeata.* Hydrobiologia, 442: 207-214.

Elliot, M., Janes, N.F., and Potter, C. (1978) The future of pyrethroids in insect control. Ann. Rev. Entomol., 23: 744-769.

Elliott, J.E., Machmer, M., Wilson, L.K., and Henny, C.J. (2000) Contaminants in Ospreys from the Pacific Northwest: II. Organochlorine pesticides, polychlorinated biphenyls and mercury, 1991-1997. Arch. Environ. Con. Tox., 38: 93-106.

Elser, J.J., and Goldman, C.R. (1990) Zooplankton effects on phytoplankton in lakes of contrasting trophic status. Limnol. Oceanogr., 36: 64-90.

Engelhardt, K.A.M., and Kadlec, J.A. (2001) Species traits, species richness and the resilience of wetlands after disturbance. J. Aquat. Plant Manage., 39: 36-39.

Favre, N., and Rudin, W. (1996) Salt-dependent performance variation of DNA polymerases in co-amplification PCR. Biotechniques, 21: 28-32.

Ferrao-Filho, A.S., and Azevedo, S. (2003) Effects of unicellular and colonial forms of toxic *Microcystis aeruginosa* from laboratory cultures and natural populations on tropical cladocerans. Aquat. Ecol., 37: 23-35.

Finizio, A., di Guardo, A., and Cartmale, L. (1998) Hazardous air pollutants (HAPS) and their effect on biodiversity: An overview of the atmospheric pathways of persistent organic pollutants (POPS) and suggestions for future studies. Environ. Monit. Assess., 49: 327-336.

Folt, C.L., Chen, C.Y., Moore, M.V., and Burnaford, J. (1999) Synergism and antagonism among multiple stressors. Limnol. Oceanogr., 44(3): 864-877.

Forbes, V.E. (2000) Is hormesis an evolutionary expectation? Funct. Ecol., 14(1): 12-24.

Freifelder, D. (1982) Physical biochemistry: Applications of biochemistry and molecular biology, Freeman W. H. and Company, USA, 494-536.

Frey, D.G. (1993) The penetration of cladocerans into saline waters. Hydrobiologia, 267: 233-248.

Friberg-Jensen, U., Wendt-Rasch, L. Woin, P., and Christoffersen, K. (2003) Effects of the pyrethroid insecticide, cypermethrin, on a freshwater community studied under field conditions. I. Direct and indirect effects on abundance measures of organisms at different trophic levels. Aquat. Toxicol., 63: 357-371.

Garcia, C. M., Garcia-Ruiz, R., Rendon, M., Niell, F. X., and Lucerna, J. (1997) Hydrological cycle and interannual variability of the aquatic community in a temporary saline lake (Fuente de Piedra, southern Spain). Hydrobiologia, 345: 131-141.

Garidel, P. (2003) Insights in the biochemical composition of skin as investigated by micro infrared spectroscopic imaging. Phys. Chem., 5: 2673-2679.

Garip, S., Bozoglu, F., and Severcan, F. (2007) Differentiation of mesophilic and thermophilic bacteria with Fourier transform infrared spectroscopy. Appl. Spectrosc., 61: 186-192.

Garip, S., Gozen, A.C., and Severcan, F. (2009) Uses of Fourier transform infrared spectroscopy for rapid comparative analysis of *Bacillus* and *Micrococcus* isolates. Food Chem., 113(4): 1301-1307.

Gee, J.H.R., Smith, B.D., Lee, K.M., and Griffiths, S.W. (1997) The ecological basis of freshwater pond management for biodiversity. Aquat. Conserv., 7(2): 91-104.

Gerten, D., and Adrian, R. (2002) Species-specific changes in the phenology and peak abundance of freshwater copepods in response to warm summers. Freshwat. Biol., 47: 2163-2173.

Giordano, M., Kansiz, M., Heraud, P., Beardall, J., Wood, B., and McNaughton, D. (2001) Fourier transform infrared spectroscopy as a novel tool to investigate changes in intracellular macromolecular pools in the marine microalga *Chaetocerosmuellerii* (Bacillariophyceae). J. Phycol., 37: 271-279.

Gliwicz, M.Z., and Sieniawska, A. (1986) Filtering activity of *Daphnia* in low concentration of a pesticide. Limnol. Oceanogr., 31(5): 1132-1138.

Gómez, A., Cecchine, G., and Snell, T.W. (1997) Effect of pentachlorophenol on predator-prey interaction of two rotifers. Aquat. Toxicol., 37: 271-282.

Gorgulu, S.T., Dogan, M., and Severcan, F. (2007) The characterization and differentiation of higher plants by Fourier transform infrared spectroscopy. Appl. Spectrosc., 61(3): 300-308.

Goulden, C.E., Comotto, R.M., Henderickson, J.A., Henry, JR. L.L., and Johnson, K.L. (1982) Procedures and recommendations for the culture and use of *Daphnia* in bioassay studies. In Aquatic toxicology and hazard assessment. 5th Conf. Am. Sot. Testing Mater. ASTM STP, 766: 148-169.

Green, J., 1993. Zooplankton associations in East African lakes spanning a wide salinity range. Hydrobiologia, 267: 249-256.

Green, J., and Menengestou, S. (1993) Specific diversity and community structure of Rotifera in a salinity series of Ethiopian inland waters. Hydrobiologia, 209: 95-106.

Grzesiuk, M., and Mikulski, A. (2006) The effect of salinity on freshwater crustaceans. Pol. J. Ecol., 54(4): 669-674.

Gunzler H., and GremlichH. U. (2002) "IR spectroscopy: An introduction" Wiley-John Wiley & Sons, Ltd.

Hall, C., and Burns, C.W. (2002) Mortality and growth responses of *Daphnia carinata* to increases in temperature and salinity. Freshwat. Biol., 47: 451-458.

Hammer, U.T. (1993) Zooplankton distribution and abundance in saline lakes of Alberta and Saskatchewan, Canada. Int. J. Salt Lake Res., 2: 111-132.

Hanazato, T. (2001) Pesticide effects on freshwater zooplankton: An ecological perspective. Environ. Pollut., 112: 1-10.

Hanazato, T. (1999) Anthropogenic chemicals (insecticides) disturb natural organic chemical communication in the plankton community. Environ. Pollut., 105: 137-142.

Hanazato, T., and Dodson, S.I. (1992) Complex effects of a kairomone of *Chaoborus* and an insecticide on *Daphnia pulex*. J. Plankton Res., 14: 1743-1755.

Hanazato, T., Fueki, K., and Yoshimoto, M. (2001) Fish-induced life history shifts in the cladocerans *Daphnia* and *Simocephalus*: Are they positive or negative responses? J. Plankton Res., 23: 945-951.

Haris, P.I., and Chapman, D. (1996) Fourier transform infrared spectroscopic studies of biomembranes systems, in: Mantsch, H. H. and Chapman, D. (Eds.) (1996) Infrared Spectroscopy of Biomolecules, Wiley-Liss, Inc., USA, 239-278.

Haris, P.I., and Severcan, F. (1999) FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media. J. Mol. Catal. B-Enzym., 7: 207-221.

Hart, B.T., Bailey, P., Edwards, R., Hortle, K., James, K., McMahon, A., Meredith, C., and Swadling, K. (1991) A review of the salt sensitivity of the Australian freshwater biota. Hydrobiologia, 210: 105-144.

Haya, K. (1989) Toxicity of pyrethroid insecticides to fish. Environ. Toxicol. Chem. 8: 381-391.

Hebert, P.D.N., and Grewe, P.M. (1985) *Chaoborus* induced shifts in the morphology of *Daphnia ambigua*. Limnol. Oceanogr., 30: 1291-1296.

Heraud, P., Stojkovic, S., Beardall, J., McNaughton, D., and Wood, B.R. (2008) Intercolonial variability in macromolecular composition in P-starved and P-replete *Scenedesmus* populations revealed by infrared microspectroscopy. J. Phycol., 44: 1335-1339.

Heraud, P., Wood, B.R., Tobin, M.J., Beardall, J., and McNaughton, D. (2005) Mapping of nutrient-induced biochemical changes in living algal cells using synchrotron infrared microspectroscopy. FEMS Microbiol. Lett., 249: 219-225.

Heugens, E. (2003) Predicting effects of multiple stressors on aquatic biota. PhD. Thesis, University of Amsterdam, The Netherlands, 167 p.

Hirschmugl, C.J., Bayarri, Z.E., Bunta, M., Holt, J.B., and Giordano, M. (2006) Analysis of the nutritional status of algae by Fourier transform infrared chemical imaging. Infrared Phys. Techn., 49(1-2): 57-63.

Holdgate, M.W. (1979) A perspective of environmental pollution. Cambridge, England: Cambridge University Press. Cited in: Mason, C.F., 2002. Biology of freshwater pollution. 4th Ed. Harlow, England: Pearson Education Limited. pp: 3.

Hrbácek, J. (1977) Competition and predation in relation to species composition of freshwater zooplankton, mainly Cladocera. In J. Cairns [ed.], Aquatic microbial communities. Garland, pp. 305-353.

Hu, S. S., Tessier, A.J. (1995) Seasonal succession and the strength of intra and interspecific competition in a *Daphnia* assemblage. Ecology, 76: 2278-2294.

International Panel on Climate Change (IPCC) Assessment Report 2007, www.ipcc.ch, last visited on August 11, 2008.

Jackson, M., and Mantsch, H.H. (1995) The use and misuse of FTIR spectroscopy in the determination of protein structure. Crit. Rev. Biochem. Mol., 30(2): 95-120.

Jak, R. (1997) Toxicant-induced changes in zooplankton communities and consequences for phytoplankton development. Ph.D Thesis, Vrije Universitat, Den Helder, The Netherlands.

Jeppesen, E., Søndergaard, M., Christoffersen, K., Theil-Nielsen, J., and Jurgens, K. (2002) Cascading trophic interactions in the littoral zone: an enclosure experiment in shallow Lake Stigsholm, Denmark. Archiv Fur Hydrobiologie, 153(4): 533-555.

Jeppesen, E., Søndergaard, M., Kanstrup, E., Petersen, B., Henriksen, R.B., Hammershøj, M., Mortensen, E., Jensen, J.P., and Have, A. (1994) Does the impact of nutrients on the biological structure and function of brackish and freshwater lakes differ? Hydrobiologia, 275-276: 15-30.

Jeppesen, E., Søndergaard, M., Pedersen, A.R., Jürgens, K., Strzelczak, A., Lauridsen, T.L., and Johansson, L.S. (2007) Salinity induced regime shift in shallow brackish lagoons. Ecosystems, 10: 47-57.

Jung, C. (2000) Insight into protein structure and protein–ligand recognition by Fourier transform infrared spectroscopy. J. Mol. Recognit., 13: 325-351.

Kačuráková, M., and Wilson, R.H. (2001) Developments in mid-infrared FT-IR spectroscopy of selected carbohydrates. Carbohyd. Polym., 44: 291-303.

Kakko, I., Toimela, T., and Taahti, H. (2003) The synaptosomal membrane bound ATPase as a target for the neurotoxic effects of pyrethroids, permethrin and cypermethrin. Chemosphere., 51: 475-480.

Kashian, D.R., and Dodson, S.I. (2002) Effects of common-use pesticides on developmental and reproductive processes in *Daphnia*. Toxicol. Ind. Health., 18: 225-235.

Kazanci, N., Toyran, N., Haris, P.I., and Severcan, F. (2001) Vitamin D_2 at high and low concentrations exert opposing effects on molecular order and dynamics of dipalmitoyl phosphatidyl choline membranes. Spectroscopy, 15: 47-55.

Kazarian, S.G., and Chan, K.L.A. (2006) Applications of ATR-FTIR spectroscopic imaging to biomedical samples. Biochim. Biophys. Acta, 1758: 858-867.

Kerfoot, W.C., and Sih, A., (1987) Predation: Direct and indirect impacts on aquatic communities. University Press of New England, Hanover, Germany, and London.

Kilham, S.S., Kreeger, D.A., Lynn, S.G., Goulden, C.E., and Herrera, L. (1998) COMBO: a defined freshwater culture medium for algae and zooplankton. Hydrobiologia, 377: 147-159.

Kim, Y., Jung, J., Oh, S., and Choi, K. (2008) Aquatic toxicity of cartap and cypermethrin to different life stages of *Daphnia magna* and *Oryzias latipes*. J. Environ. Sci. Heal. B., 43: 56-64.

Kinder, R., Ziegler, C., and Wessel, J.M. (1997) γ-Irradiation and UV-C light-induced lipid peroxidation: A Fourier transform-infrared absorption spectroscopic study. Int. J. Radiat. Biol., 71(5): 561-571.

Kong, J., and Yu, S. (2007) Fourier transform infrared spectroscopic analysis of protein secondary structures. Acta Bioch. Biop. Sin., 39(8): 549-559.

Krimm, S., and Bandekar, J. (1986) Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. Adv. Protein. Chem., 38: 181-364.

Lampert, W. (1985) Food limitation and the structure of zooplankton communities. Arch. Hydrobiol. Beih. Ergebn. Limnol., 21: 497.

Lampert, W. (1987) Feeding and nutrition in *Daphnia*, p. 143–192. *In* R.H. Peters and R. De Bernardi [eds.], *Daphnia*. Memorie dell'lstituto Italiano di Idrobiologia Dott. Marco de Marchi, Vol. 45. Consiglio Nazionale delle Ricerche, Istituto Italiano di Idrobiologia.

Lampert, W. (1993) Phenotypic plasticity of the size at first reproduction in *Daphnia* - the importance of maternal size. Ecology, 74: 1455-1466.

Larsson, P., and Miracle, M.R. (1997) The future of cladoceran research. Hydrobiologia. 360, 287-289.

Larsson, P., and Dodson, S. (1993) Invited review – chemical communication in planktonic animals. Arch Hydrobiol, 129: 129-155.

Lauridsen, T., and Buenk, I. (1996) Diel changes in the horizontal distribution of zooplankton in the littoral zone of two shallow eutrophic lakes. Arch. Hydrobiol., 137: 161-176.

Li, X., Ping, X., Xiumei, S., Zhenbin, W., and Liqiang, X. (2005) Toxicity of cypermethrin on growth, pigments, and superoxide dismutase of *Scenedesmus obliquus*. Ecotox. Environ. Safe., 60: 188-192.

Lignot, J. H., Spanings-Pierrot, C.and , Charmantier, G. (2000) Osmoregulatory capacity as a tool in monitoring the physiological condition and the effect of stress in crustaceans. Aquaculture, 191: 209-245.

Lisk, D.J. (1991) Environmental effects of landfills. Sci. Total Environ. 100, 415-468. Cited in: Mason, C.F., 2002. Biology of freshwater pollution. 4th Ed. Harlow, England: Pearson Education Limited. pp: 23.

Liu, K., Bose, R., Mantsch, H.H. (2002) Infrared spectroscopic study of diabetic platelets. Vib. Spectrosc., 28: 131-136.

Loose, C.J. (1993) *Daphnia* diel vertical migration behavior: Response to vertebrate predator abundance. Ergeb. Limnol., 39: 29-36.

Loose, C.J., and Dawidowicz, P. (1994) Trade-offs in diel vertical migration by zooplankton: the costs of predator avoidance. Ecology, 80: 2348-2357.

Loose, C.J., von Elert, E., and Dawidowicz, P. (1993) Chemically-induced diel vertical migration in *Daphnia*: a new bioassay for kairomones exuded by fish. Arch. Hydrobiol., 126: 329-337.

Luz, B.R. (2006) Attenuated total reflectance spectroscopy of plant leaves: a tool for ecological and botanical studies. New Phytol., 172: 305-318.

Lynch, M. (1978) Complex interactions between natural co exploiters – *Daphnia* and *Ceriodaphnia*. Ecology, 59: 552-564.

Lynch, M. (1980) Aphanixomenon blooms: Alternate control and cultivation by *Daphnia pulex*. Am. Sot. Limnol. Oceanogr. Spec. Symp., 3: 299-304.

Machácek, J. (1991) Indirect effect of planktivorous fish on the growth and reproduction of *Daphnia galeata*. Hydrobiologia, 225: 193-198.

Machácek, J. (1995) Inducibility of life-history changes by fish kairomone in various developmental stages of *Daphnia*. J. Plankton Res., 17: 1513-1520.

Martínez-Jerónimo, F., Martínez-Jerónimo, L. (2007) Chronic effect of NaCl salinity on a freshwater strain of *Daphnia magna* (Straus) (Crustacea: Cladocera): A demographic study. Ecotoxicol. Environ. Saf., 67: 411-416.

Mason, C.F. (2002) Biology of freshwater pollution. 4th Ed. Harlow, England: Pearson Education Limited.

Matthiessen, P., Sheahan, D., Harrison, R., Kirby, M.,Rycroft, R., Turnbull, A., Volkner, C., and Williams, R. (1995) Use of a *Gammarus pulex* bioassay to measure the effects of transient carbofuran run-off from farmland. Ecotox. Environ. Saf., 30: 111-119.

Matveed, V., Matveeva, L., and Jones, A.G. (2000) Relative impacts of *Daphnia* grazing and direct simulation by fish on phytoplankton abundance in mesocosm communities. Freshwat. Biol., 44: 375-385.

Maul, J.D., Farris, J.L., and Lydy, M.J. (2006) Interaction of chemical cues from fish tissues and organophosphorous pesticides on *Ceriodaphnia dubio* survival. Environ. Pollut., 141: 90-97.

Maund, S.J., Hamer, M.J., Warinton, J.S., and Kedwards, T.J. (1998) Aquatic ecotoxicology of the pyrethroid insecticide lamda-cyhalothrin: consideration for higher-tier aquatic risk assessment. Pestic. Sci., 54: 408-417.

Maund, S.J., Sherratt, T.N., Stickland, T., Biggs, J., Williams, P., Shillabeer, N., and Jepson, P.C. (1997) Ecological considerations in pesticide risk assessment for aquatic Ecosystems. Pestic. Sci., 49: 185-190.

Mayer, F.L., and Ellersieck, M.R. (1986) Manual of acute toxicity: Interpretation and database for 410 chemicals and 66 species of freshwater organisms. Washington DC: U.S. Fish and Wild Life Service.

McKelvy, M.L., Britt, T.R., Davis, B.L., Gillie, J.K., Lentz, L.A., Leugers, A., Nyquist, R.A., and Putzig, C.L. (1996) Infrared spectroscopy. Anal. Chem., 68 (12): 93-160.

McWilliam, R.A., and Baird, D.J. (2002) Postexposure feeding depression: A new toxicity endpoint for use in laboratory studies with *Daphnia magna*. Environ. Toxicol. Chem., 21: 1198-1205.

Mecozzi, M., Pietroletti, M., and Mento, R. (2007) Application of FTIR spectroscopy in ecotoxicological studies supported by multivariate analysis and 2D correlation spectroscopy. Vib. Spectrosc., 44: 228-235.

Medina, M., Barata, C., Telfer, T., and Baird, B.J. (2004) Effects of cypermethrin on marine plankton communities: a simulated field study using mesocosms. Ecotox. Environ. Safe, 58: 236-245.

Meems, N., Steinberg, C.E.W., and Wiegand, C. (2004) Direct and interacting toxicological effects on the waterflea (*Daphnia magna*) by natural organic matter, synthetic humic substances and cypermethrin. Sci. Total Environ., 319: 123-136.

Mendelsohn, R. and Mantsch, H. H. Fourier transform infrared studies of lipid-protein interaction, in: Watts, A and De Pont, J. J. H. H. M. (Eds.) (1986) Progress in Protein-Lipid Interactions, Volume 2, Elsevier Science Publishers, Netherlands, 103-147.

Mian, L.S., and Mulla, M.S. (1992) Effects of pyrethroid insecticides on non-target invertebrates in aquatic ecosystems. J. Agric. Entomol., 9: 73-98.

Mikulski, A. (2001) The presence of fish induced the quick release of offspring by *Daphnia*. Hydrobiologia, 442: 195-198.

Millennium Ecosystem Assessment Report 2005, www.maweb.org/en/index.aspx, last visited on August 9, 2008.

Ministry of Agriculture and Rural Affairs, General Directorate of Protection and Control Statistics (2004).

Moss, B. (1998) Ecology of Freshwaters. Man and Medium, Past to Future, third ed. Blackwell Science, UK.

Newton, I. and Wyllie, I. (1992) Recovery of a sparrowhawk population in relation to declining pesticide contamination. J. Appl. Ecol., 29: 476-484.

OECD (1984) Part I: 24h acute immobilisation test. OECD Guideline for Testing of Chemicals, 16p.

OECD (1998) OECD guideline for testing chemicals 211. *Daphnia magna* reproduction test. Organization of Economic Co-operation and Development, Paris, France.

OECD (2004) *Daphnia* sp., acute immobilization test. In: OECD guidelines for testing of chemicals, vol. 202. Paris: Organization for Economic Cooperation and Development.

Ohkawa, H., Kikuchi, R., and Miyamoto, J. (1980) Bioaccumulation and biodegradation of the (S)-acid isomer of fenvalerate (Sumicidin) in an aquatic model ecosystem. J. Pest. Sci. 5: 11-22. From: Pieters Prof Dissertation 2007.

Padovani, L., Capri, E. and Trevisan, M. (2004) Landscape-level approach to assess aquatic exposure via spray drift for pesticides: A case study in a Mediterranean area. Environ. Sci. Technol., 38(12): 3239-3246.

Palaniappan, PL.RM., Krishnakumar, N., and Vadivelu, M. (2008) FT-IR study of the effect of lead and the influence of chelating agents, DMSA and D-Penicillamine, on the biochemical contents of brain tissues of *Catla catla* fingerlings. Aquat. Sci., 70: 314-322.

Patel, S.A., Currie, F., Thakker, N., and Goodacre, R. (2008) Spatial metabolic fingerprinting using FT-IR spectroscopy: investigating abiotic stresses on *Micrasterias hardyi*. Analyst., 133: 1707-1713.

Patel, S.A., Pandey, A. K., Bajpayee, M, Parmar, D., and Dhawan, A. (2006) Cypermethrin-induced DNA damage in organs and tissues of the mouse: Evidence from the comet assay. Mutat. Res., 607: 176-183.

Pereira, J.L., and Gonçalves, F. (2007) Effects of food availability on the acute and chronic toxicity of the insecticide methomyl to *Daphnia* spp. Sci. Total Environ., 386: 9-20.

Pieters, B.J., and Liess, M. (2006) Population developmental stage determines the recovery potential of *Daphnia magna* populations after fenvalerate application. Environ. Sci. Technol., 40: 6157-6162.

Pieters, B.J., Jager, T., Kraak, M.H.S., and Admiraal, W. (2006) Modeling responses of *Daphnia magna* to pesticide pulse exposure under varying food conditions: intrinsic versus apparent sensitivity. Ecotoxicology., 15: 601-608.

Pijanowska, J. (1997) Alarm signals in *Daphnia*? Oecologia., 112: 12-16.

Polat, H., Erkoc, F.U., Viran, R. and Kocak, O. (2002) Investigation of acute toxicity of beta-cypermethrin on guppies *Poecilia reticulate*. Chemosphere, 49: 39-44.

Ramdani, M., Elkhiati, N., and Flower, R. J. (2001) Open water zooplankton communities in north African wetland lakes: the CASSARINA project. Aquat. Ecol., 35: 319-333.

Ratushnyak, A.A., Andreeva, M.G., and Trushin, M.V. (2005) Effects of type II pyrethroids on *Daphnia magna*. Riv. Biol.-Biol. Forum., 98: 349-358.

Reede T. (1995) Life history shifts in response to different levels of fish kairomones in *Daphnia*. J. Plankton Res., 17: 1661-1667.

Relyea, R., and Hoverman, J. (2006) Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. Ecol. Lett., 9: 1157-1171.

Reynaldi, S., and Liess, M. (2005) Influence of duration of exposure to the pyrethroid fenvalerate on sublethal responses and recovery of *Daphnia magna* Straus. Environ. Toxicol. Chem., 24: 1160-1164.

Reynaldi, S., Duquesne, S., Jung, K., and Liess, M. (2006) Linking feeding activity and maturation of *Daphnia magna* following short-term exposure to fenvalerate. Environ. Toxicol. Chem., 25: 1826-1830.

Ricciardi, A., and Rasmussen, J. B. (1999) Extinction rates of North American freshwater fauna. Conserv. Biol., 13: 1220-1222.

Richman, S. (1958) The transformation of energy by *Daphnia pulex*. Ecol. Monogr., 28: 273-291.

Ringelberg, J. (1991) A mechanism of predator-mediated induction of vertical migration in *Daphnia hyaline*. J. Plankton Res., 13: 83-89.

Rinke, K., Hülsmann, S., and Mooij, W.M. (2008) Energetic costs, underlying resource allocation patterns, and adaptive value of predator-induced life-history shifts. Oikos, 117: p273-285.

Saha, S. and Kaviraj, A. (2003) Acute Toxicity of Synthetic Pyrethroid Cypermethrin to Freshwater Catfish Heteropneustes fossilis (Bloch). Int. J. Toxicol., 22: 325-328.

Sakamoto, M., Chang, K., and Hanazato, T. (2006) Inhibition of development of anti-predator morphology in the small cladoceran *Bosmina* by an insecticide: impact of an anthropogenic chemical on prey–predator interactions. Freshwater Biol., 51: 1974-1983.

Sakwinska, O. (1998) Plasticity of *Daphnia magna* life history traits in response to temperature and information about a predator. Freshwater Biol., 39: 681-687.

Sakwinska, O. (2002) Response to fish kairomone in *Daphnia galeata* life history traits relies on shifts to earlier instar at maturation. Oecologia, 131: 409-417.

Sala, O. E., Chapin III, F. S., Armesto, J. J., Berlow, E., Bloomfield, J., and 14 others (2000) Global Biodiversity Scenarios for the Year 2100. Science, 287.

Sánchez, P., and Tarazona, J.V. (2002) Development of a multispecies system for testing reproductive effects on aquatic invertebrates. Experience with *Daphnia magna*, *Chironomus prasinus* and *Lymnaea peregra*. Aquat. Toxicol., 60: 249-256.

Sarma, S.S.S., and Nandini, S. (2006) Review of recent ecotoxicological studies on cladocerans. J. Environ. Sci. Heal. B., 41(8): 1417-30.

Sarma, S.S.S., Nandini, S., Morales-Ventura, J., Delgado-Martinez, I., and Gonzalez-Valverde, L (2006) Effects of NaCl salinity on the population dynamics of freshwater zooplankton (rotifers and cladocerans Aquat. Ecol., 40: 349-360.

Sarnelle, O. (1997) *Daphnia* effects on microzooplankton: Comparisons of enclosure and whole-lake responses. Ecology, 78 (3), 913-928.

Schallenberg, M., Hall, C.J., and Burns, C.W. (2003) Consequences of climate-induced salinity increases on zooplankton abundance and diversity in coastal lakes. Mar. Ecol. - Prog. Ser., 251: 181-189.

Schulz, R., and Liess, M. (1999) A field study of the effects of agriculturally derived insecticide input on stream macroinvertebrate dynamics. Aquat. Toxicol., 46: 155-176.

Scullion, J., Elliott, G.N., Huang, W.E., Goodacre, R., Worgan, H., Darby, R., Bailey, M.J., Gwynn-Jones, D., Griffith, G.W., Winson, M.K., Williams, P.A., Clegg, C., and Draper, J. (2003) Use of earthworm casts to validate FT-IR spectroscopy as a 'sentinel' technology for high-throughput monitoring of global changes in microbial ecology. Pedobiologia, 47: 440-446.

Segal-Rosenheimer, M., and Dubowski, Y. (2007) Heterogeneous ozonolysis of cypermethrin using real-time monitoring FTIR techniques. J. Phys. Chem. C. 111(31): 11682-11691.

Severcan, F., Gorgulu, G., Gorgulu, S.T., and Guray, T. (2005) Rapid monitoring of diabetes-induced lipid peroxidation by Fourier transform infrared spectroscopy: Evidence from rat liver microsomal membranes. Anal. Biochem., 339: 36-40.

Sheffield, S.R. and Lochmiller, R.L. (2001) Effects of field exposure to diazinon on small mammals inhabiting a semienclosed prairie grassland ecosystem. I. Ecological and reproductive effects. Environ. Toxicol. Chem., 20(2): 284-296.

Sigee, D.C., Dean, A., Levado, E., and Tobin, M.J. (2002) Fourier-transform infrared spectroscopy of *Pediastrum duplex*: characterization of a micro-population isolated from a eutrophic lake. Eur. J. Phycol, 37(1): 19-26.

Simsek Ozek, N., Sara, Y., Onur, R., and Severcan, F. (2009) Low dose simvastatin induces compositional, structural and dynamical changes on rat skeletal extensor digitorum longus muscle tissue. Bioscience Reports. *In press*. doi:10.1042/BSR20080150.

Smith, T.M., and Stratton, G.W. (1986) Effects of synthetic pyrethroid insecticides on non-target organisms. Residue Rev., 97: 93-120.

Soderlund, D.M., Clark, J.M., Sheets, L.P., Mullin, L.S., Piccirillo, V.J., Sargent, D., Stevens, J.T., and Weiner, M.L. (2002) Mechanisms of pyrethroid neurotoxicity: Implications for cumulative risk assessment. Toxicology, 171: 3-59.

Soucek, D.J. (2007) Bioenergetic effects of sodium sulfate on the freshwater crustacean, *Ceriodaphnia dubia.* Ecotoxicology, 16: 317-325.

Souillac, P.O., Middaugh, C.R., and Rytting, J.H. (2002) Investigation of protein/carbohydrate interactions in the dried state. 2. Diffuse reflectance FTIR studies. Int. J. Pharm., 235: 207-218.

Soyab, A.P., Currie, F., Thakker, N., and Goodacre, R. (2008) Spatial metabolic fingerprinting using FT-IR spectroscopy: investigating abiotic stresses on *Micrasterias hardyi*. Analyst., 133: 1707-1713.

Spaak, P. and Boersma, M. (1997) Tail spine length in *Daphnia galeata* complex: costs and benefits of induction by fish. Aquat. Ecol., 31: 89-98.

Spaak, P. and Boersma, M. (2001) The influence of fish kairomones on the induction and vertical distribution of sexual individuals of the *Daphnia galeata* species complex, Hydrobiologia, 442: 185-193.

Spaak, P., Vanoverbeke, J. and Boersma, M. (2000) Predator-induced life-history changes and the coexistence of five taxa in a *Daphnia* species complex. Oikos, 89: 164-174.

Stansfield, J. Moss, B. and Irvine, K. (1989) The loss of submerged plants with eutrophication III. Potential role of organochlorine pesticides: a palaeoecological study. Freshwater Biol., 22: 109-132.

Stearns, S. (1992) The evolution of life histories. Oxford University Press, New York.

Stehfest, K., Toepel, J., and Wilhelm, C. (2005) The application of micro-FTIR spectroscopy to analyze nutrient stress-related changes in biomass composition of phytoplankton algae. Plant Physiol. Bioch. 43 (7): 717-726.

Stephenson, R.R. (1982) Aquatic toxicology of cypermethrin. I. Acute toxicity to some freshwater fish and invertebrates in laboratory tests. Aquat.Toxicol. 2 (3), 175-185.

Stibor, H. (1992) Predator-induced life-history shifts in a freshwater cladoceran. Oecologia, 192: 162-165.

Stich, H.B., and Lampert, W. (1981) Predator evasion as an explanation of diurnal vertical migration. Nature, 293: 396-398.

Strecker, U., Faúndez, V., and Wilkens, H. (2004) Phylogeography of surface and cave *Astyanax* (Teleostei) in Upper Central America based on cytochrome b sequence data. Mol. Phylogenet. Evol., 33: 469-481.

Stuart, B. (1997) Biological applications of infrared spectroscopy. John Wiley and Sons, Chichester.

Stuart, B. (2004). In: Infrared Spectroscopy, Fundamentals and Applications.

Taub, F.B. (1997) Unique information contributed by multispecies systems: Examples from the standardized aquatic microcosm. Ecol. Appl., 7(4): 1103-1110.

Teschner, M. (1995) Effects of salinity on the life history and fitness of Daphnia magna: variability within and between populations. Hydrobiologia, 307: 33-41.

Thomas, C., Cameron, D., Green, A., Bakkenes, R. E., Beaumont, M., Collingham, L. J., Erasmus, B. F. N., De Siqueira, Y. C., Grainger, M. F., Hannah, A., Hughes, L. L., Huntley, B., Van Jaarsveld, A. S., Midgley, G. F., Miles, L., Ortega- Huerta, M. A., Peterson, A. T., Phillips, O. L., and Williams, S. E., 2004. Extinction risk from climate change. Nature, 427: 145-148.

Thomas, C.R., Hose, G.C., Warne, M.St J., and Lim, R.P. (2008) Effects of river water and salinity on the toxicity of deltamethrin to freshwater shrimp, cladoceran, and fish. Arch. Environ. Contam. Toxicol., 55: 610-618.

Tollrian, R., and Dodson, S.l. (1999) Inducible defences in cladocera, constraints, costs, and multi-predator environments. in Tollrian & Harvell (eds) The ecology and evolution of inducible defenses. Princeton University Press, Princeton New Jersey.

Toyran, N., Lasch, P., Naumann, D., Turan, B., and Severcan, F. (2006) Early alterations in myocardia and vessels of the diabetic rat heart: An FTIR microspectroscopic study. Biochem. J. 397: 427-436.

Tripathi, P.K., and Singh, A. (2004) Toxic effects of cypermethrin and alphamethrin on reproduction and oxidative metabolism of the freshwater snail, *Lymnaea acuminata*. Ecotox. Environ. Safe, 58: 227-235.

Urabe, J., Clasen, J., and Sterner, R.W. (1997) Phosphorus limitation of *Daphnia*: Is it real? Limnology and Oceanography, 42: 1436-1443.

U.S. Environmental Protection Agency (EPA) ECOTOX Database, http://cfpub.epa.gov/ecotox, last visited on June 2009.

U.S. Environmental Protection Agency (2008) Reregistration eligibility decision for cypermethrin; Doc. No. EPA-HQ-OPP-2005-0293-0036. U.S. EPA, Washingtom D.C., USA: Revised Version of June, 2006.

U.S. Environmental Protection Agency (2002a) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms; 5th Ed.; Doc. No. EPA-821-R-02-012, U.S. EPA, Washington D.C., USA.

U.S. Environmental Protection Agency (2002b) Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms; 4th Ed.; Doc. No. EPA-821-R-02-013. U.S. EPA, Washington D.C., USA.

van der Schalie, W.H., and Gentile, J.H. (2000) Ecological risk assessment: implications of hormesis. J. Appl. Toxicol., 20: 131-139.

Van Dijk, H.G. and Guicherit, R. (1999) Atmospheric dispersion of current-use pesticides: A review of the evidence from monitoring studies. Water Air Soil Poll., 115: 21-70.

Van Donk, E., Prins, H., Voogd, H.M., Crum, S.J.H. and Brock, T.C.M. (1995) Effects of nutrient loading and insecticide application on the ecology of *Elodea*-dominated freshwater microcosms. I. Responses of plankton and zooplanktivorous insects. Arch. Hydrobiol., 133: 417-439.

van Gool, E., and Ringelberg, J. (2002) Relationship between fish kairomone concentration in a lake and phototactic swimming by *Daphnia*. J. Plankton Res. 24 (7), 713-721.

Van Wijngaarden, R.P.A., T.C.M. Brock, and P.J. Van Den Brink. (2005) Threshold levels for effects of insecticides in freshwater ecosystems: A review. Ecotoxicology, 14: 355-380.

Vandenbrouck, T., Soetaert, A., van der Ven, K., Blust, R., and De Coen, W. (2009) Nickel and binary metal mixture responses in *Daphnia magna*: Molecular fingerprints and (sub)organismal effects. Aquat. Toxicol., 92: 18-29.

Vijveberg, H.P.M., and de Weille, J.R. (1985) The interaction of pyrethroids with voltage-dependent Na channels. Neurotoxicology, 6: 23-34.

Vijverberg, J. (1989) Culture techniques for studies on the growth, development and reproduction of copepods and cladocerans under laboratory and in situ conditions: A review. Freshwater Biol., 21(3): 317-373.

Visman, V., Mcqueen, D.J. and Demers, E. (1994) Zooplankton spatial patterns in two lakes with contrasting community structure, Hydrobiol., 284: 177-191.

von Elert, E. (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. Limnol. Oceanogr., 47: 1764-1773.

von Elert, E. and Stibor, H. (2006) Predator-mediated life history shifts in *Daphnia*: enrichment and preliminary chemical characterisation of a kairomone exuded by fish. Arch. Hydrobiol., 167: 21-35.

Wagner, A., and Benndorf, J., 2007. Climate-driven warming during spring destabilises a *Daphnia* population: a mechanistic food-web approach. Oecologia, Vol. 151, pp. 351-364.

Wallace, R.L., and Snell, T. W. (2001) Phylum Rotifera. In: Thorp JH, Covich AP (eds) Ecology and classification of North American freshwater invertebrates. Academic Press, 2nd edn. New York, pp 195-254.

Warren, N., Allan, I.J., Carter, J.E., House, W.A. and Parker, A. (2003) Pesticides and other micro-organic contaminants in freshwater sedimentary environments – a review. Appl. Geochem., 18: 159-194.

Weider, L.J. and Pijanowska, J. (1993) Plasticity of *Daphnia* life histories in response to chemical cues from predators, Oikos, 67: 385-392.

Wendt-Rasch, L., Friberg-Jensen, U., Woin, P., and Christoffersen, K. (2003) Effects of the pyrethroid insecticide cypermethrin on a freshwater community studied under field conditions. II. Direct and indirect effects on the species composition. Aquat. Toxicol., 63: 373-389.

Williams, W.D. (1987) Salinization of rivers and streams: an important environmental hazard. Ambio., 16: 180-185.

Winder, M., and Schindler, D. E., 2004. Climatic effects on the phenology of lake processes. Global Change Biol., 10: 1844-1856.

World Resource Institute, www.wri.org, last visited on June 21, 2005.

Zala, S.M., and Penn, D.J. (2004) Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. Anim. Behav., 68: 649-664.

Zaret, T.M. (1980) Predation and freshwater communities. Yale Univ. Press, New Haven, Connecticut., pp. 187.

Zaret, T.M., and Suffern, J. S. (1976) Vertical migration in zooplankton as a predator avoidance mechanism. Limnol. Oceanogr., 21: 804-813.

Zhang, H., Ishikawa, Y., Yamamoto, Y., and Carpentier, R. (1998) Secondary structure and thermal stability of the extrinsic 23 kDa protein of photosystem II studied by Fourier transform infrared spectroscopy. FEBS Letters, 426: 347-351.

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name:	Akkaş, Sara Banu
Nationality:	Turkish (TC)
Date and Place of Birth:	29 November 1980, ABD
Marital Status:	Single
Phone / Fax:	+90 312 210 11 47 / +90 312 210 11 51
E-mail:	banuakkas@yahoo.com
Foreign Languages:	English (Native Speaker), French (Elementary)

EDUCATION

Degree	Institution	Graduation	C.GPA
Ph.D.	METU Biology Dept.	2009	3.75
M.Sc.	METU Biology Dept.	2003	3.57
B.Sc.	METU Biology Dept.	2001	3.29
High School	METU Development Foundation High School, Ankara	1997	

WORK EXPERIENCE

Year	Place	Enrollment
2008-Present	TROYKA Ltd. METU Technopolis, Ankara	International Projects Expert
February, 2005	Donald Bren School of Environmental Science & Management UC Santa Barbara, USA	Invited Researcher
2001-2008	METU Biology Department	Research Assistant
Summer, 2000	Department of Biophysics School of Medicine Ankara Univ. Supervisor: Prof. Dr. Belma Turan	Internship in Electrophysiology

TEACHING EXPERIENCE

Middle East Technical University Student Union Election Committee Member

Coordination of assistants' activities

BIO 714 "Introduction to Freshwater Biology Laboratory" Assistantship

BIO 321 "Bioanalytical Chemistry Laboratory" Assistantship

BIO 112 and BIO 501 "Seminars in Biology for Freshman and Graduate Students" Assistantship

GENE 475 "Molecular Biology Laboratory" Assistantship

GENE 270 "Experimental Techniques in Molecular Biology Laboratory" Assistantship

GENE 103 "Molecular and Cellular Biology I Laboratory" Assistantship

ACADEMIC ACTIVITIES

Academic Publications

Akkas, S.B., Beklioglu, M., Severcan, F. Molecular approach to the effects of pesticide and fish predation on *Daphnia*: An ATR-FTIR Spectroscopic Study. *Manuscript in preparation*.

Bezirci, G., Akkas, S.B., Yildirim, F., Severcan, F., Beklioglu, M. Impacts of salinity and fish-exuded kairomone on the survival and molecular profile of *Daphnia*. *Manuscript submitted to Aquatic Toxicology*.

Akkas, S.B., Bezirci, G., Yildirim, F., Severcan, F., Beklioglu, M. Effects of pesticide and fish predation on *Daphnia pulex*: Organismal and molecular approach. *Manuscript submitted to Aquatic Toxicology*.

Akkas, S.B., Kepenek, A.O., Beklioglu, M., Severcan, F. Molecular approach to the chemical characterization of fish-exuded kairomone: A Fourier transform infrared spectroscopic study. *Manuscript accepted by Aquatic Sciences*.

Beklioglu, M., Akkas, S.B., Ozcan, H.E., Bezirci, G., Togan, I. Effects of 4-nonylphenol, fish predation and food availability on survival and life history traits of *Daphnia magna* straus. *Manuscript to be submitted to Ecotoxicology and Environmental Safety*.

Akkas, S.B., Severcan, M., Yilmaz, O., Severcan, F. (2007) Effects of lipoic acid supplementation on rat brain tissue: An FTIR and neural network study. *Food Chem*. 105 (3): 1281-1288. **Times Cited: 10**

Akkas, S.B., Inci, S., Zorlu, F., Severcan, F. (2007) Melatonin affects the order, dynamics and hydration of brain membrane lipids. *J. Mol. Struct*. 834-836: 207-215. **Times Cited: 1**

Academic Research Projects

"Design of a Model and an Index Predicting the Status of an Ecosystem by Determining the Effects on the Bioindicator *Daphnia* of Salinity and Insecticide Quantity in Lakes" An Interdisciplinary Project
Funded by: TÜBİTAK-ÇAYDAG-104Y308
Principal Investigator: Prof. Dr. Meryem Beklioğlu
Investigators: Prof. Dr. Feride Severcan; Res. Assist. S. Banu Akkaş
Duration: July, 2005 - September, 2007 (Completed)

"Design of a Fast Dependable Fish Stock Determination Method Based on Kairomones Released from Fish" An Interdisciplinary Project
Funded by: TÜBİTAK-YDABAG-100Y035
Principal Investigator: Assoc. Prof. Meryem Beklioğlu
Investigators: Prof. Dr. Feride Severcan; Assist. Prof. Dr. Ayşegül Ozan
Duration: September, 2000 - September, 2003 (Completed)

"Investigation of the Effects of Antioxidants on Brain Tissue and Membrane at the Molecular Level by Biophysical Techniques"
Funded by: Middle East Technical University
Principal Investigator: Prof. Dr. Feride Severcan
Duration: May, 2002 - May, 2003 (Completed)

International Conferences, Workshops and Seminars Attended

July 8-13, 2007	SEFS 5 - Symposium for European Freshwater Sciences Poster Presentation Palermo, Italy
September 3-8, 2006	"XXVIII. European Congress on Molecular Spectroscopy" Poster Presentation Istanbul, Turkey
September 3-9, 2005	"VII. International Symposium on Cladocera" (with Partial Stipend) Poster Presentation Herzberg, Switzerland
September 2-3, 2005	"7th Pre-Conference Subfossil Cladocera Workshop" Herzberg, Switzerland
July 15-17, 2005	"1st WSEAS International Conference: Cellular and Molecular Biology – Biophysics and Bioengineering" Poster Presentation Athens, Greece

February 12-16, 2005	"49th Annual Meeting of Biophysical Society" Poster Presentation Long Beach, California, USA
March, 22-23, 2004	"Modern Biology and Visions of Humanity" International Encounter organized by the European Commission's Group on Life Sciences Invited Participant Genoa, Italy
February 14-18, 2004	"48th Annual Meeting of Biophysical Society" Poster Presentation Baltimore, Maryland, USA
October 12-15, 2003	"13th Balkan Biochemical Biophysical Days & Meeting on Metabolic Disorders" Oral Presentation Aydın, Turkey
April 22 - May 3, 2002	"Workshop on Theoretical Ecology: Natural Resource Management and Conservation Biology" International Workshop (with Full Stipend) The Abdus Salam International Centre for Theoretical Physics Trieste, Italy
November 28-30, 2001	"Shallow Lake Wetlands: Ecology, Euthrophication, and Restoration" International Workshop - Preparation Team Middle East Technical University Ankara, Turkey

National Conferences, Workshops and Seminars Attended

August 27-29, 2008	"III. National Limnology Symposium" Poster Presentation Faculty of Fisheries, Ege University İzmir, Turkey
June 26-30, 2006	"XVIII. National Biology Conference" Poster Presentation Adnan Menderes University Aydın, Turkey

September 19-22, 2004	"XVI. National Biophysics Conference" Poster Presentation Hacettepe University Ankara, Turkey
September 18-19, 2004	"Optophysiology and Confocal Microscopy Course" Faculty of Medicine, Hacettepe University Ankara, Turkey
October 8-12, 2003	"XV. National Biophysics Conference" Poster Presentation Pamukkale University Denizli, Turkey
March, 2003	"Red Lists, Priority Species and National Conservation Action Plans" National Workshop Turkish Bird Research Society Middle East Technical University Ankara, Turkey
January 31, 2003	European Union Sixth Framework Program Food Quality and Safety Risks 3rd National Workshop Ankara University Ankara, Turkey
September 4-7, 2002	"XVI. National Biology Conference" Poster Presentation İnönü University Malatya, Turkey
June, 2002	Protein Crystallography & Structural Biology Seminars Turkish Crystallography Society Hacettepe University Ankara, Turkey
October 10-13, 2001	"8th National Biology Student Conference" Hacettepe University Ankara, Turkey

Academic Fellowships

Fall, 2005 – 2008	Ph. D. Fellowship from The Scientific and Technological Research Council of Turkey (TÜBİTAK)

PROFESSIONAL ACTIVITIES

May 11-12, 2009	European Union FP7-NMP SKINTREAT Management Board Meeting "Novel approaches for the development of customized skin treatments and services (Test case: Dead Sea Minerals and Conventional Drugs)" (213202) Trondheim, Norway
May 7-8, 2009	European Union FP7-KBBE GMSAFOOD Management Board Meeting "Biomarkers for post market monitoring of short and long-term effects of genetically modified organisms (GMOs) on animal and human health" (211820) Fermoy, Ireland
April 21-22, 2009	European Union FP6-SME LOWJUICE Management Board Meeting "Novel process for reducing sugar and adding fibre to natural apple juices for increased public health and increased competitiveness of the European fruit juice industry" (30379) Madrid, Spain
April 20-21, 2009	European Union FP6-SME BARLEYBREAD Management Board Meeting "European guideline for healthy high fibre/ low salt baking process based on the use of European barley" (30269) Madrid, Spain
December 2-3, 2008	European Union FP7 IncoNet EECA Brokerage Event in Food, Agriculture and Fisheries, and Biotechnology Invited Participant Astana, Kazakhstan

September 15-16, 2008	European Union FP7-NMP SkinTreat Kick-Off Meeting "Novel approaches for the development of customized skin treatments and services (Test case: Dead Sea Minerals and Conventional Drugs)" (213202) Gembloux, Belgium
June 3-5, 2008	Workshop on Project Development for the funding programs for R&D activites of private companies settled in Turkey funded by the *Technology and Innovation Funding Programs Directorate* (TEYDEB) of The Scientific and Technological Research Council of Turkey (TÜBİTAK) Organized by METU Technopolis Ankara, Turkey
April 17-18, 2008	"Security Research Conference - Technology solutions to enhance systems interoperability" Organized by TÜBİTAK Ankara, Turkey

HOBBIES: She has been a member of the "Evolutionary Working Group" in Turkey since 2006. She has been doing translations, editing and voice recordings for the last ten years in several fields ranging from journal articles to English language exams. She was a guide for the foreign participants of the 32nd International Physics Olympiad held in Antalya, Turkey during the summer of 2001. She has been playing the piano since 1988. She enjoys reading in various topics and travelling around the world and Turkey.