COMBINED EFFECTS OF THE 4-NONYLPHENOL AND FISH KAIROMONES ON THE SURVIVAL, MORPHOLOGY AND LIFE HISTORY TRAITS OF *Daphnia magna* Straus

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ABSTRACT

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Daphnia has evolved morphological and life history defenses against predators and these appear to be mediated by chemicals (kairomones) released by the predator. Furthermore, anthropogenic stressors including alkylphenols have been shown to affect several characteristics of *Daphnia*.

Daphnia magna that were grown in the fish-conditioned water and nonfish-conditioned water were exposed to 0.005, 0.01, 0.05, 0.15, 0.5 mg l^{-1} NP concentrations in the acute toxicity and 0.001, 0.005, 0.01 mg l^{-1} NP concentrations in the chronic toxicity experiments. In the chronic toxicity experiment, two different food levels were used.

The 24 and 48h LC_{50} values of NP for the individuals were determined as 0.394 mg l^{-1} and 0.149 mg l^{-1}, respectively in the presence of fish kairomone.

However, in the absence of fish kairomone LC_{50} values could not be calculated due to high survival rate. In the chronic toxicity experiment, fish kairomone and NP significantly decreased the survival of the test organisms compared to the effects of either treatment. The effect of fish kairomone was stronger than the NP concentrations; however, at the highest NP concentration the effect was pronounced. Presence of fish kairomone and NP together significantly increased the maturation time whereas fish kairomone itself significantly decreased the maturation size. The high food level and the NP doses increased the helmet size, and the former also increased clutch size.

In conclusion, exposure to fish kairomones enhanced the sensitivity to the NP doses, which were very low in this study, through reducing the survival and affecting the life history characteristics.

Key words: *Daphnia*, alkylphenol, nonylphenol, fish kairomone

ÖZ

4-NONİL FENOL VE BALIK SİNYALİNİN *Daphnia magna* Straus'UN YAŞAMA ORANI, MORFOLOJİSİ VE POPULASYON PARAMETRELERINE OLAN ETKİLERİ

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Daphnia, avcı balıklardan salgılandığı düşünülen kimyasallar vasıtasıyla avcılarına karşı morfolojik, davranışsal ve populasyon parametrelerini içeren birçok savunma yöntemleri geliştirmiştir. Aynı zamanda, alkil fenollerin de *Daphnia*'nın bu özelliklerini etkilediği bilinmektedir.

Balık sinyalli suda ve control suyunda yetişen bireyler akut toksisite deneyinde 0.005, 0.01, 0.05, 0.15, 0.5 mg l^{-1} NF konsantrasyonlarına, kronik toksisite deneyinde ise 0.001, 0.005, 0.01 mg l^{-1} NF konsantrasyonlarına tabi tutulmuştur. Kronik toksisite deneyinde ayrıca 2 farklı besin konsantrasyonu kullanılmıştır.

Balık sinyaline maruz bırakılmış bireylerde 24 ve 48 saatlik LC_{50} değerleri sırasıyla 0.394 mg l^{-1} ve 0.149 mg l^{-1} olarak bulunmuştur. Ancak, balık sinyaline

maruz bırakılmayan bireylerde bu değerler hesaplanamamıştır. Kronik toksisiti deneyinde NF ve balık sinyaline tabi tutulan bireylerde yaşam oranı bu iki faktörün tek başına etkisine kıyasla belirgin bir biçimde düşmüstür. Balık sinyalinin etkisi NF'ün etkisinden daha kuvvetli olup, bu etki en yüksek NF konsantrasyonunda daha belirgindir. Bu durumda, NF ve balık sinyalinin etkilerinin birbirini kuvvetlendirdiği söylenebilir. NF ve balık sinyalinin bireylerin erginleşme süresini belirgin bir biçimde arttırdığı görülürken, tek başına balık sinyali bireylerin ergin boylarında düşmeye neden olmuştur. Yüksek besin miktarı ve NF bireylerin baş uzunluğunu arttırmış, besin miktarının yüksek olması ayrıca birey başına üretilen yumurta sayısında da artışa sebebiyet vermiştir.

Sonuç olarak, balık sinyali bu çalışmada çok düşük konsantrasyonlarda kullanılan NF'e karşı bireylerin yaşama oranlarının düşmesi ve populasyon parametrelerinin değişmesi yoluyla belirgin bir hassasiyet oluşturmuştur.

Anahtar kelimeler: *Daphnia*, alkil fenol, nonil fenol, balık sinyali.

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To My Parents

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CHAPTER 1

INTRODUCTION

1.1. *Daphnia*, as a model organism for toxicological studies

Daphnia (or Daphnids) are members of a collection of animals that are broadly termed as "water fleas". These are small crustaceans and belong to a family known as Daphniidae, which in turn is part of the Cladocera, relatives of the freshwater shrimp, Gammarus spp, and the brine shrimp, Artemia spp. They get their common name from their jerky movement through the water. The taxonomy of the *Daphnia* was given in Figure 1 (Macan, 1959).

Group: Eukaryota

Kingdom: Metazoa

Phylum: Arthropoda

Class: Crustacea

Group: Branchiopoda

Order: Cladocera

Family: Daphniidae

Genus: Daphnia

Figure 1 : Taxonomy of *Daphnia*.

Daphnia, feed on particles found floating in the water namely phytoplankton but they also feed on phytoplankton attached to vegetation or found on decaying organic material. However, their predominant foods are free-living algae (e.g. Chlamydomanas spp, Volvox spp, etc), bacteria and fungi (Moss, 1998). In the summer months, they can often be seen in very high density in ponds and lakes as the concentration of algae builds up. Their prolificity is due to a great extent to their ability to replicate by parthenogenicity (Lampert and Sommer, 1996).

Daphnia tend to be almost kidney shaped, possessing only a single compound eye though they have an ocellus, a simple eye, two doubly-branched antennae with about half the length of the body or more, and leaf-like limbs inside the carapace that produce a current of water which carries food and oxygen to the mouth and gills. Their bodies are almost transparent (Figure 2 and 3).

A carapace covers the body, including the 4 to 6 pairs of thoracic appendages, and is used as a brood chamber (Figure 2 and 3). The abdomen and post-abdomen (distal to the anus) is generally bent forward under the thorax. The post-abdomen bears two large claws used primarily for cleaning debris out of the carapace. Swimming is accomplished by downward strokes of the large second antennae (Macan, 1959).

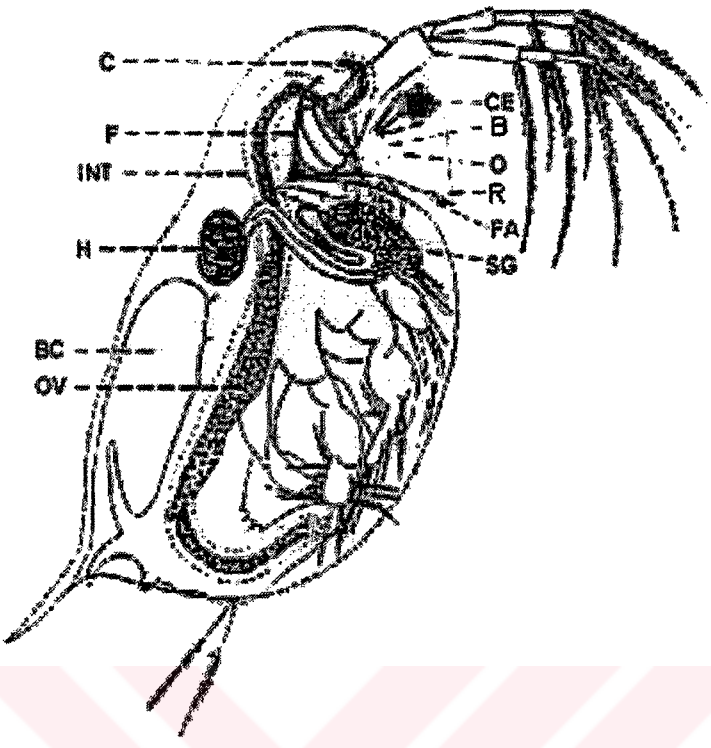

Figure 2: Anatomy of female *Daphnia pulex* (De Geer) (greatly magnified); diagrammatic; (muscles not shown in fig 2). *B*, brain; *BC*, brood chamber; *C*, digestive caecum; *CE,* compound eye; *F*, fornix; *FA*, first antenna (antennule); *H*, heart; *INT*, intestine; *O*, ocellus.

In most species complex movements of the thoracic appendages produce a constant current of water between the valves. Small particles (less than 50 microns in diameter) in the water are filtered out by fine setae on the thoracic legs and moved along a groove at the base of legs to the mouth. Although there is some evidence that certain types of food, such as particular types of algae, Protozoa, or bacteria may be selected by some species, it is generally believed that all organic particles of suitable size are ingested without any selective mechanism. When undesirable material or large tangled masses are introduced between the mandibles, they may be removed by spines on the first legs and then kicked out of the carapace by the post-abdomen (Macan, 1959).

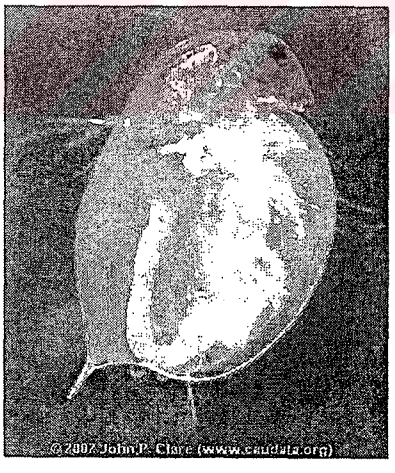

Figure 3: Photograph of a female *Daphnia magna* (taken from John P. Clare).

Adult *Daphnia* range in size from half a millimeter to almost a centimeter, depending on the species, though within a given species, size can vary greatly (female *Daphnia magna* can be between 2.5 mm and 4 mm).

Parthenogenicity is the ability to self-replicate without fertilization of any form that is a type of asexual reproduction. The offspring are exact genetic replicas of the parent clones, and any differences in the physical state of the clones are due to environmental conditions (Dodson and Frey, 1991). Parthenogenesis seems to have evolved to allow *Daphnia* to take advantage of good conditions e.g. food, temperature, etc as soon as they arise. In natural habitats, during the late spring, summer and early autumn depending on temperature, food availability and presence of waste products of their metabolism, *Daphnia* reproduce by parthenogenicity, bearing, on the average, ten live young per individual. Furthermore, the entire race is made up of females during this period (Lynch, 1980). Developing embryos are often visible in the mother's body without the aid of a microscope.

When environmental conditions are unfavourable including food scarcity, intensive predation pressure, low water temperature some eggs develop into males and the females produce eggs that must be fertilized. These eggs develop into small embryos which then go into suspended animation, and are shed with the carapace as dark brown/black saddle-shaped cases known as ephippia (ephippium is Latin for saddle) (Shurin and Dodson, 1997). These can survive harsh conditions and are quite capable of withstanding a dry spell if their pond dries up for a while, and they can sometimes even survive freezing. The ephippial females of most daphnids are easy to tell from their live-bearing counterparts because the developing ephippium is visible

as a black spot towards the rear end of the animal. When conditions improve again, the egg producing generations begin producing live young once again (all females), and the male sex dies out completely until it is needed when conditions worsen once again (Dodson and Frey, 1991).

The production of ephippia is essential to the maintenance of many *Daphnia* populations. Ephippia represent a bet-hedging reproductive strategy to deal with environments that become inhabitable. Populations in seasonal habitats are reestablished yearly from banks of resting eggs, and, even in populations that overwinter, hatching resting eggs make up a significant portion of the juveniles born in the spring (Wolf and Carvalho, 1989). Ephippia are also frequent vectors of dispersal between lakes (Mellors, 1975).

The life span of *Daphnia*, from the release of the egg into the brood chamber until the death of the adult, is highly variable depending on the species and environmental conditions (e.g. water temperature, predation, and food availability). Generally the life span increases as temperature decreases due to lowered metabolic activity. The average life span of *D. magna* is about 40 days at 25°C and about 56 days at 20°C.

Four distinct periods may be recognized in the life history of *Daphnia*: (1) egg, (2) juvenile, (3) adolescent and (4) adult (Lynch, 1980). Typically, a clutch of 6 or 10 eggs is released into the brood chamber. The eggs hatch in the brood chamber and the juveniles, which are already similar in form to the adults, are released in approximately two days when the female molts (casts off her exoskeleton). The time required to reach maturity varies from 6 to 10 days. *Daphnia* typically invest most of

their energy in reproduction (69% in *D. magna,* 67% in D. pulex), while they invest comparatively little in growth (23%). This serves to highlight the heavy emphasis on fast reproduction to take advantage of good conditions (Lynch, 1980).

Daphnia are widely used in the study of behavioral ecology, evolution and aquatic ecotoxicology because of their rapid clonal reproduction and extreme sensitivity to their chemical environment. Firstly, *Daphnia* are a key component of many lentic systems. They are the major consumers of primary production (Lampert and Sommer, 1996) and flagellated protozoans, and in turn, they are major food source for vertebrate and invertebrate predators. Therefore, deleterious effects on *Daphnia* may cascade both upwards and downwards through the aquatic food web (Barry and Stoopman, 2000). Secondly, *Daphnia* are paradigmatic species, and effects on daphnids are often correlated with responses in other species (Barry and Stoopman, 2000). Finally, daphnids have a wide range of inducible phenotypes which are sensitive to environmental pollutants (Hanazato, 1999). These responses provide a tool for investigations on the effect of toxic chemicals on phenotypic plasticity in general.

1.2. Effect of fish predation on *Daphnia*

It has long been considered that predation is one of the major factors influencing zooplankton community structure (Brooks and Dodson, 1965; Gliwicz, 1994). Planktivorous fish perform size-selective predation, feeding on larger, more conspicuous prey, Daphnia (Zaret and Kerfoot, 1975). This may cause a reduction in mean individual size and the size at maturity in zooplankton populations, and a shift

in the community structure from one dominated by large-bodied cladocerans to one dominated by small-bodied cladocerans and rotifers (Brooks and Dodson, 1965; Lynch, 1979).

There is growing body of evidence that indirect effects of fish predation, induced at the individual level, may also influence the temporal and spatial distribution of zooplankton (Riessen and Sprules, 1990). Zooplankton has evolved a variety of morphological, life history and behavioral defenses against predators and these appear to be mediated by chemicals released by the predator (Dodson, 1984). These chemicals have been termed kairomones, and are defined as substances that give benefit to the receivers rather than the releasers (Brown et. al., 1970).

Although the precise nature of these substances is currently uncertain, preliminary chemical characterization of kairomones exuded by fish has indicated that the active component is a low-molecular-weight, water soluble, non-volatile substance that is stable over a wide temperature and pH range (Loose et. al., 1993; Von Elert and Loose, 1996). Importantly, kairomones released by different fish species exhibit the same chemical characteristics, suggesting that these kairomones are similar, if not identical (Von Elert and Loose, 1996; Von Elert and Pohnert, 2000). It has been suggested that bacteria associated with fish are responsible for the production of "fish kairomone" (Ringelberg and Van Gool, 1998), although it is possible that the experimental conditions employed may have resulted in an overestimation of the bacterial contribution to the release of the kairomone (Von Elert and Pohnert, 2000).

Studies have shown that fish kairomones may cause short-term changes in cladoceran swarm behavior and swimming patterns (Jensen et. al., 1998; Seely and Lutnesky, 1998), and may induce diel vertical migration if water depth allows (Loose et. al., 1993; Stirling, 1995; Van Gool and Ringelberg, 1998). Changes in swarm behavior and swimming patterns have been interpreted as short-term behavioral adaptations to minimize vulnerability to predation (Jensen et.al., 1998; Seely and Lutnesky, 1998). Diel vertical migration is generally viewed as a strategy for avoiding predators that rely on sight to catch their prey (Lampert, 1993; Larsson and Dodson, 1993).

The presence of kairomones from vertebrate predators has also been shown to induce changes in the life history characteristics of cladocerans. Cladocerans experiencing strong selective pressure by fish, either directly by exposure to fish or indirectly via exposure to their kairomones, have been shown to shift their reproductive strategy to produce many but small neonates (Dodson, 1989; Stibor, 1992; Weider and Pijanowska, 1993; Hanazato, 1995; Machacek, 1995; Reede, 1995). Some species also appear to reach sexual maturity at a smaller size in the presence of fish or fish kairomones (Vanni, 1987; Dodson, 1989; Leibold and Tessier, 1991; Tessier et.al., 1992; Vonder Brink and Vanni, 1993; Hanazato, 1995), which may allow individuals to produce offspring before they become vulnerable to fish predation. The production of large numbers of offspring is ecologically advantageous if predation pressure is strong, as it is increases the chance of offspring surviving to reproduce. These changes are viewed as adaptive responses to fish predation, as they reduce the risk of predation.

1.3. Effect of food on *Daphnia*

Food quantity varies considerably both spatially and temporally in lakes (Andersen and Hessen, 1991). Herbivores must, therefore, be able to cope with fluctuating food concentrations in order to survive. *Daphnia* can adjust their filtering rates to the changes in available food to maximize food intake at all times. They can also develop larger filter screens (Egloff and Palmer, 1971; Stuchlik, 1991; Lampert, 1994) within which more particles can be retained, or adjust the mesh size of the filter screens to enable them to retain smaller particles (Hartmann and Kunkel, 1991).

Variation in food condition affects several traits of *Daphnia* life history because organisms allocate energy to growth and reproduction in such a way as to optimize the sum of present and future reproduction (Williams, 1966). The allocation of energy within the animal is affected by the total amount available. A specific life history strategy that is advantageous at high food conditions might not be beneficial at low food conditions. Since most life history traits are plastic with respect to food availability during the development of *Daphnia* (Cashwell, 1983), the life history strategy can be altered. Under limiting food conditions, a reduction in somatic growth rate, later maturation and a reduced reproductive output have been observed (Threlkeld, 1976; Lynch, 1989, 1992; McCauley et.al., 1990). An increase in body size has also been found and was shown to decrease the risk of starvation (Riessen and Sprules, 1990). Neglecting the aspect of food availability will therefore results in a failure of detect important aspects of phenotypic plasticity which is thought to contribute to survival in a changing environment.

Therefore, it is necessary to learn more about the interactions among biotic factors such as kairomones indicative for predation threat, and the food concentration, indicative for starvation threat.

1.4. Relationship between kairomone and toxic chemicals

Aquatic organisms in the natural environment often are exposed to natural stresses (kairomones, starvation, high and low temperature, low oxygen conditions) and toxic chemicals (pesticides, alkylphenols, metals) simultaneously. These two kinds of stressors may have complex effects on the organisms, sometimes being antagonistic or additive, and other times synergistic (Hanazato, 2001).

Natural stressors such as predation, food shortage, oxygen deficiency, and high temperature can affect pesticide toxicity. Folt et. al. (1999) tested the effects of sodium dodecyl sulfate at low food availability and high temperature on *Daphnia*. He found that a combination of these stressors was more harmful than either one alone. Hanazato and Dodson (1995) demonstrated that predator kairomone, low oxygen concentration and an insecticide can reduce the population fitness of *Daphnia* synergistically.

A good example of zooplankton being exposed to both antropogenic and natural stressors is the diel vertical migration (DVM) of *Daphnia* in a pesticide-contaminated lake. In a regular DVM, the animals migrate downward into dark waters in the morning to avoid fish predation and upwards in the evening to feed on algae, which are a primary source of their food (Lampert, 1994). Fish kairomone is a strong cue for inducing the DVM of *Daphnia* (Lampert and Loose, 1992), even when

they must migrate downward into water that is low in oxygen and lacking in food resources. During DVM, *Daphnia* experience stress from oxygen and food deficiency, and they become more sensitive to pesticides than expected from laboratory toxicity tests.

Hanazato and Dodson (1992) have found that Chaoborus (an invertebrate, phantom midge larvae) kairomone synergistically increased the toxicity of carbaryl (a pesticide) and prolonged the mainenance of neckteeth in *D. pulex*.

However, when *D. longicephala* were simultaneously exposed to sublethal concentrations of carbaryl and kairomone released from a notonectid (invertebrate predator), the crest size of 3-day-old daphnids was decreased at 3.2 $\mu g\ l^{-1}$ carbaryl and the kairomone reversed the effects of carbaryl on the age, body length and brood size at maturity (Barry, 1999).

Controversy surrounded on the effect of fish kairomone on the clutch size in the literature. Dodson (1989), Stibor (1992), Weider and Pijanowska (1993) found an increase in clutch size in response to the fish kairomone. Whereas, reduced clutch size was found in response to the fish kairomone in the other studies (Havel and Dodson, 1987; Black and Dodson, 1990; Hanazato and Dodson, 1992).

Presence of predator kairomone may confer benefit to *Daphnia*, which is a reduction of mortality in the presence of the predator. However, *Daphnia*'s response to the kairomone is achieved at a cost in the form of a reduced tolerance to environmental stress such as high water temperature, food shortage and low oxygen concentration (Hanazato, 1991a; Hanazato, 1991b.; Hanazato and Dodson, 1995).

Furthermore, natural stressors may alter the sensitivity of zooplankton to toxic chemicals including pesticides, alkylphenols. Moreover, zooplankton species in the natural environment may be more sensitive to toxic chemicals (alkylphenols, pesticides) than the same species cultured under favourable conditions in the laboratory. Thus standard tests may underestimate toxicity (Hanazato, 2001).

1.5. Endocrine disrupting chemicals

Many chemicals have been shown to be capable of mimicking or interfering with the reactions of hormones in animals and people. This compounds are called as "endocrine disrupting chemicals" (EDCs) (Barry and Stoopman, 2000).

Environment Agency (2000) has defined EDCs as naturally occurring or synthetic substances that interfere with the functioning of endocrine systems resulting in unnatural responses. The observed possible effects of EDC's are given in Table 1.

Table 1: Possible effects of EDC's

Males	**Females**	**Wildlife**
Decreased sperm counts or quality	Breast cancer	Infertility
Testicular cancer	Cardiovascular effects	Sex changes (imposex)
Undescended testes	Intelligence deficit	Developmental abnormalities
Malformed penis	Neurological problems	Thyroid disfunction
Intelligence deficit		Behavioral abnormalities
Neurological problems		Disfucntional immune system

4-Nonylphenol is one of the EDC's, a toxic degradation product of alkylphenol ethoxylates (APE) which are nonionic surfactants used in a variety of products including institutional cleaning agents, textiles, agricultural chemicals, plastics, paper products, house-hold cleaning agents, and personal care products (Talmage, 1994).

Alkylphenol ethoxylate surfactants are usually made from a branched-chain nonylphenol or octylphenol, reacted with ethylene oxide. An APE molecule consists of two parts (Figure 4) The alkylphenol portion of an APE molecule consists of a hydrocarbon chain attached to phenol. The alkylphenol portion gives APEs the ability to dissolve grease and other substances that are not soluble in water. The ethoxylate portion of an APE is a long chain of two-carbon units connected by oxygen atoms. This structure makes APEs soluble in water and helps remove dirt and grease from soiled surfaces into water (APE Research Council: White Paper, 1995).

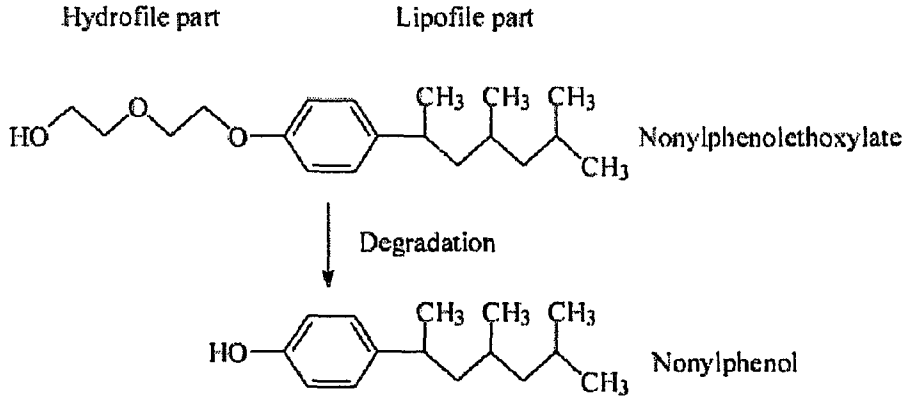

Figure 4: The structure of nonylphenol and nonylphenol ethoxylates which is an example of alkylphenol·ethoxylates.

The toxicity of alkylphenols usually increased as the length of the hydrophobic chain increases (McLeese et. al., 1981). Toxicity may occur by partition into lipid membranes in the organism, for example the mitochondrial membrane, leading to the uncoupling of energy production (Argese et. al., 1994).

The most widely used APEs in commerce are nonylphenol ethoxylates (NPEs), in which the alkylphenol portion of the structure consists of a 9-carbon side chain.

Over one billion Sterlin of nonylphenol ethoxylates are produced annually (Talmage, 1994) Nonylphenols have been detected in aquatic environments throughout the United States and Europe (Marcomini and Giger, 1987; Grifoll et. al., 1990; Ahel et. al., 1994, Naylor, 1995). In a study of 30 rivers in the United States, nonylphenol was found to be ubiquitous with the highest measured aqueous concentration of 0.64 μg/L (Naylor, 1995). Aqueous concentrations as high as 5 and 45μg/L have been measured in Boston Harbor, Massachusettes, USA and Glatt River, Switzerland (Ahel et. al., 1994), respectively. Aqueous concentrations in the Glatt River were generally between 1 and 10 μg/L (Ahel et.al., 1994).

1.6. Effects of Alkylphenols on organisms

Alkylphenols were first found to be oestrogenic (oestrogen-mimicking) in the 1930s (Dodds and Lawson, 1938), and more evidence was published in 1978 (Mueller and Kim, 1978).

Oestrogenic effects have been shown in rainbow trout hepatocytes, chicken embryo fibroblasts and a mouse oestrogen receptor (Jobling and Sumpter, 1993; White et al., 1994).

Adult male rainbow trout exposed to 30μg l^{-1} of either octylphenol, nonylphenol or nonylphenoxy acetic acid (NP1EC), similar to levels found in UK Rivers, produce the female egg yolk protein vitellogenin (Ashfield et al, 1995). Another experiment, examining the effects of exposure to 30μg l^{-1} of OP, NP, NP1EC and NP2EO in male rainbow trout has shown a reduction in testicular growth and OP can increase vitellogenin production when present in the water at only 4.8μg l^{-1} (Jobling et al., 1996).

A survey of wild fish (roach) in UK Rivers found that a high percentage of males had eggs in their testes, in addition to female egg yolk protein in their blood (Jobling et.al., 1998). It is believed that alkylphenol ethoxylates may be largely responsible for this effect in UK Rivers polluted with industrial effluent (such as the River Aire).

Miles and Richardson et al. (1999) studied effects on male and female fathead minnows exposed to 4-*p* nonylphenol (4-NP, branched) at concentrations of 0.05, 0.16, 0.4, 1.6 or 3.4 mg/L (measured values) for 42 days. As a result, abnormality in testis tissue was observed, among males in the group exposed to 1.6 mg/L or higher in the tissue examination using electron microscope.

Kahl et al (1997) studied effects on egg lumps of the midges exposed to 4-nonylphenol (4-NP, branched, according to manufacturer) at concentrations of 8, 18,

36, 84 or 138 μg/L for 20 days. They recorded morphogenic abnormality in the group exposed to 36 μg/L or more.

1.7. Effects of nonylphenol on *Daphnia*

Results presented by Baldwin et.al. (1997), demonstrate that exposure of daphnids to 4-nonylphenol caused an elevation in the accumulation of testosterone.

The 24 and 48 h EC_{50} values of nonylphenol, NP, to *D. magna*, based on immobilization, were determined as 0.30 (0.26-0.35) and 0.19 (0.17-0.21) mg/l (Comber et al., 1993). The most sensitive 21 day no observed effect concentration (NOEC) of NP to *D. magna* was found to be 0.024mg/l which was about one order magnitude below the acute 48 h EC_{50} value.

In the study by Shurin and Dodson (1997), prenatal exposure to nonylphenol caused a non-lethal but disabling abnormality in *Daphnia* developing from embryos into juveniles. The deformity was seen in 11% of live young grown at 10μg/L nonylphenol, below the NOEC of nonylphenol for *D. magna* (24μg/L). This concentration was within the range commonly found in waters that receive sewage effluent (Blackburn and Waldock, 1995; Kvestak and Ahel, 1994).

Furthermore, Dodson and Hanazato (1995) found that the yearly maximum percentage of males in populations of three *Daphnia* species in Lake Mendota, USA, decreased drastically from 1885 to 1975. They hypothesized that anthropogenic chemicals, which began to be widely used in the 1940s, altered the *Daphnia* sex ratio in the lake through their hormone-like effects.

The impact of natural stressors with anthropogenic stressors have not been widely explored. The combined effect of the insecticide carbaryl and the *Chaoborus* (an invertebrate) kairomone on helmet development in *Daphnia ambigua* was studied by Hanazato (1995). It is founded that *Daphnia* developed helmets in response to kairomone, but not in response to carbaryl at low (sublethal) concentrations (1-3 $\mu g\ l^{-1}$). However, the carbaryl enhanced the development of high helmets and prolonged the maintenance period of the helmets over instars in the presence of kairomone. These results suggest that sublethal concentrations of the insecticide alter predator-prey interactions by inducing helmet formation in *Daphnia*, which may reduce vulnerability of the *Daphnia* to predation. The study of Hanazato and Dodson (1992) showed that the kairomone of *Chaoborus* made the *Daphnia pulex* more sensitive to the insecticide carbaryl. Also, potential population growth rate of *D. pulex* was reduced synergistically by the kairomone of *Chaoborus* and the insecticide.

1.8. Scope of the study

Impacts of nonylphenol and fish kairomone have not been studied before. In this study we took the adventage of such gap in the literature to explore the such antropogenic and natural stressors effect together.

In this study, the four questions given below were investigated.

(i) What is the acute effect of NP on *D. magna*?

(ii) Is there a synergistic or antagonistic acute effect of NP and fish cue on *D. magna*?

(iii) What are the changes on morphology and life history traits when animals exposed to only fish cue, only NP, and fish cue and NP simultaneously?

(iv) What is the effect of low food level and high food level on morphology and life history traits when animals exposed to only fish cue, only NP, and fish cue and NP simultaneously in the chronic toxicity experiment?

CHAPTER 2

MATERIAL AND METHODS

2.1.Test organism

A clone of *Daphnia magna* Straus originally was collected from Lake Eymir, Ankara and was cultured in the laboratory conditions since 1999 and used in the experiments. The specimens were maintained in dechlorinated tap water, identical to dilution water, in a climate room with a temperature of 21±1 °C and a photoperiod of 16h light: 8h dark. The cultures were fed with a defined diet of fresh culture of algae, *Scenedesmus obliquus* three times a week.

The pure culture of *S.obliquus* of fresh food was obtained from Göttingen University Algal Culture Centre and continuously cultured in the same climate room. *S. obliquus* were cultured both as liquid and solid forms using proteose peptone medium. For the preparation of liquid proteose peptone medium, for about a 1 l^{-1} medium, 10 ml of each K_2HPO_4, $MgSO_4.7H_2O$, NaCl, $NaNO_3$, $CaCl_2.2H_2O$, KH_2PO_4, 940 ml distilled water, 1 g proteose-peptone were used (see Appendices 1). Solid medium was prepared following the same procedure but 15 g agar for 1 liter of fresh algal medium. was added. The culture was renewed every twenty days. Inoculations were realized in sterile laminar flow hood to prevent any contamination.

Both solid and liquid cultures were kept in the same climate room with 21±1 °C and 16h light: 8h dark photoperiod under 3000 lux. The cultures were kept on magnetic stirrers to give agitation.

Fish used in the experiments to produce kairomones was bleak, (*Alburnus alburnus* L.) and also collected from Lake Eymir and reared in the aquariums in the same climate room. They were fed with fish food and live *Daphnia*. The water in the aquariums were discharged and refilled with dechlorinated tapwater once a week.

2.2.Preparation of test solutions

2.2.1. Nonylphenol solutions

4-Nonylphenol was purchased from the Sigma-Aldrich laboratories. Stock solutions of 4-nonylphenol (NP) were prepared separately by dissolving weighed amounts of NP in ethanol. Test solutions were prepared by the addition of appropriate aliquots of stock solution to dilution water using a micropipette stirring by magnetic stirrer. Amount of ethanol in stock solutions were equalized as all test concentrations and the solvent control contained 100 µl of ethanol per liter. The control consisted of distilled water only.

2.2.2.Fish-conditioned water

To prepare fish- conditioned water, two individuals of bleak were reared in the 10 l of aged and dechlorinated tapwater for 24 hours. After the incubation period, the water was filtered through Whatman GF/C filter to remove any larger particles. Then the fish-conditioned water for the experiments was ready to use.

2.3. Experimental procedures

Egg-bearing *Daphnia* which had been reared in the aquariums, when they had stage1 eggs (Threlkeld, 1979) in their brood chamber, were transferred to the aged and dechlorinated tapwater, in which fish had never been reared (control water). According to the Threlkeld (1979) , stage 1 is an egg stage that egg membrane intact and there was no differentiation into body regions. Conversely, another set of egg-bearing specimens were transferred to the fish-conditioned water. When the *Daphnia* in these sets release their neonates, which is three days after transferring, the neonates were ready to use in the experiments.

2.3.1. Acute toxicity experiment

The neonates of *D. magna* (≤ 24h-old) which were obtained from the egg-bearing individuals reared in the absence of fish kairomone or presence of fish kairomone were exposed to a range of concentrations of NP for 56h under controlled conditions of the climate room (21±1 °C and 16h light:8h dark photoperiod). The concentrations of NP tested were 0.005, 0.01, 0.05, 0.15 and 0.5 mg l^{-1}. For the

control, solvent control (100µl l^{-1} ethanol) and the each test concentration given above 5 *D. magna* were added to glass vessels containing 50 ml of relevant test solution. The animals were not fed during the course of the test. The survival of the animals was assessed by examining from 9:00 a.m. to 17:00 a.m., once in two hours. The experiment contains 4 replicates per treatment (Table 2).

Table 2: The plan of the acute toxicity experiment

	(50 ml)		(50 ml) NP concentrations				
	Control	Solvent control	0.005 mg/l	0.01 mg/l	0.05 mg/l	0.15 mg/l	0.5 mg/l
Neonates reared in the absence of fish kairomone	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)
Neonates reared in the presence of fish kairomone	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)	5 neonate n:4 (4x5)

2.3.2. Chronic toxicity experiment with varying food concentrations

The neonates (≤24 h-old) which obtained from the egg-bearing individuals rearing in the control water or fish-conditioned water were exposed to a range of concentrations of NP and 2 sets of food levels which included 1mg C l^{-1} as high food level and 0.075mg C l^{-1} as low food level) under controlled conditions in the climate room with renewal of test solutions and food concentrations every 48 hours. The concentrations of NP tested included 0.001, 0.005 and 0.01 mg/l. For the control,

solvent control (100µl/l ethanol) and each test concentration one *D. magna* was held in glass vessels containing 50 ml of relevant test solution. The experiment contained five replicate per treatment (Table 3).

Table 3: The plan of the chronic toxicity experiment with varying food concentrations

		(50 ml)		(50 ml) NP concentrations		
		Control	Solvent control	0.001 mg/l	0.005 mg/l	0.01 mg/l
High food level	Neonates reared in the absence of fish kairomone	1 neonate n:5	1 neonate n:5	1 neonate n:5	1 neonate n:5	1 neonate n:5
Low food level	Neonates reared in the absence of fish kairomone	1 neonate n:5	1 neonate n:5	1 neonate n:5	1 neonate n:5	1 neonate n:5
High food level	Neonates reared in the presence of fish kairomone	1 neonate n:5	1 neonate n:5	1 neonate n:5	1 neonate n:5	1 neonate n:5
Low food level	Neonates reared in the presence of fish kairomone	1 neonate n:5	1 neonate n:5	1 neonate n:5	1 neonate n:5	1 neonate n:5

During the experiment animals were examined daily and following parameters were measured using a dissecting microscope the precision of ±0.1 mm (Figure 5):

- length of the animal

  x: apex of helmet to eye

  y: eye to base of tail spine

- survival: number of individuals living per treatment
- egg/ individual : number of eggs per one female bearing (clutch size)
- maturation time: time that a neonate requires to become an egg-bearing individual.
- maturation size: size of the adult individual

Figure 5. The morphometric parameters measured in the chronic toxicity experiment.

The adjustment of the appropriate food level was explained below.

2.3.2.1. Preparation of the food

Chlorophyll-a content of the algal culture was determined using a method described by Jesper and Christoffersen (1989). According to the method, 90 ml of dechlorinated tapwater was added to the 10 ml of liquid algal culture and filtered

through 47 mm Whatman GF/C filter paper. The filter paper was then placed in a centrifuge tube and incubated in 10 ml of ethanol (96%) for 12 hours in complete dark and then centrifugated in 4000 rpm for 15 minutes. The absorbance of the supernatant was measured at 750 nm and 663 nm against an ethanol blank. The chlorophyll-a concentration was calculated according to the equation below:

$$\textbf{Chlorophyll}_{a}\ (\mu g/l) = (11.0 \times (A_{663}-A_{750}) \times V_{ethanol}) / V_{filtered\ water}$$

Where: A_{663} is the absorbance at 663 nm

A_{750} is the absorbance at 750 nm

$V_{ethanol}$ is the volume of ethanol blank in ml

$V_{filtered\ water}$ is the volume of the filtered water in liter

11.0 is the coefficient.

Then the concentration of chlorophyll-a was converted to C amount using the conversion factor of 30 mg C: 1 mg chl-a l^{-1} suggested by Reynolds (1984). The C amount in the experimental vessels was adjusted to 1 mg C l^{-1} and 0.075 mg C l^{-1} as high and low food levels, respectively.

2.4. Statistical methods

SAS and SPSS package programs were used for the statistical evaluation of the data. Determination of the LC_{50} values was performed with the help of the Minitab software program.

CHAPTER 3

RESULTS

3.1.Acute toxicity experiment

In the acute toxicity study, *D. magna* which were grown in fish-conditioned water and control water (dechlorinated tapwater) were exposed to a range of concentrations of nonylphenol for 56 h under static conditions at the climate room. The concentrations tested were 0.005, 0.01, 0.05, 0.15 and 0.5 mgl^{-1}. There were 4 repeats for each treatment. The survival of the animals was assessed by observing and recording the number of death from 9:00 am. to 17:00 am. once in two hours. The experiment lasted for 56 hours.

To test the effects of NP concentartions and fish kairomone on survival of the individuals, repeated measures of ANOVA (General Linear Model, SAS package program) was performed. The test revealed that all the effects were higly significant (Table 4).

Table 4: Results of Repeated measures of ANOVAs for the effect of the treatments (fish kairomone, NP concentrations) on survival of *Daphnia magna*.

Treatment	DF	F	Significance level
NP	6	325.73	< .0001
Fish	1	39.28	< .0001
NP * Fish	6	9.78	< .0001
Error	42		
Time	13	107.3	< .0001
Time * NP	78	39.45	< .0001
Time * Fish	13	15.45	< .0001
Time * NP * Fish	78	2.16	< .0001
Error	546		

Results can be visualized with the help of figures as will be presented. The cumulative average number of death for each treatment which was composed of 4 replicates containing 5 individuals in each test container for each time interval throughout the experiment is shown in Figure 6 a and b. Figure 6 a shows the cumulative average number of death of the individuals which were grown in non-fish condition at the presence of different NP concentrations, and Figure 6 b shows the individuals were grown in the fish-conditioned water at the presence of different NP concentrations. Without effect of fish kairomone, there was no death in the treatments of 0.05, 0.01, 0.005 mgl^{-1}NP, control (c) and solvent control (sc) (Figure 6a). The death was observed only in the 0.5 and 0.15 mgl^{-1}NP treatments. The presence of significant NP dose effect on the survival was already indicated in Table

4. However, in the presence of fish kairomone, there was an increase in the number of death per test container at 0.15 mgl^{-1}NP concentration. Moreover, death was observed even in the 0.05, 0.01 and 0.005 mgl^{-1}NP concentrations of the experiment (Figure 6b). The cumulative average death was very high in 0.5 mgl^{-1}NP in the absence and presence of fish kairomone. The maximum cumulative death was reached within the 26 hours in both cases.

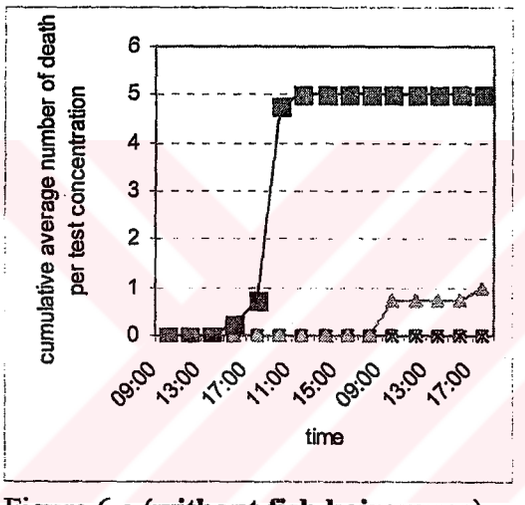

Figure 6 a (**without fish kairomone**)

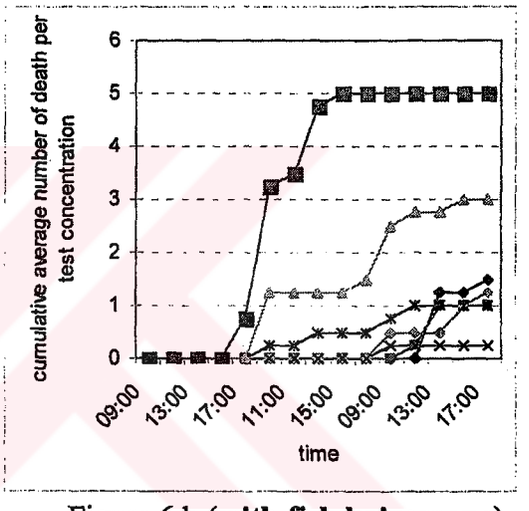

Figure 6 b (**with fish kairomone**)

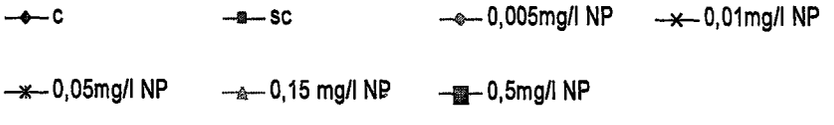

Figure 6: Cumulative average number of death of the individuals grown in a) different NP concentrations b) different NP concentrations but now in the presence of fish kairomone.

As these figures 6a and b show, there was a difference for survival rate between the individuals grown in different conditions in presence/absence of fish kairomone at different NP level. The presence of this difference was manifested by the repeated measures of ANOVA, the fish kairomone on the survival of *D. magna* was to be found significant (Table 4).

The 24 and 48h LC_{50} values of NP for the individuals grown in the fish-conditioned water were determined as 0.494 mg l^{-1} (ranging from 0.31 to 0.51 mg^{-1}) and 0.149 mg l^{-1} (ranging from 0.111 to 0.243 mg l^{-1}), respectively. However, LC_{50} values for the individuals that were reared in the absence of fish kairomone could not be calculated due to the inappropriate data.

To have a better understanding of the effects of NP doses in the presence and absence of fish kairomone Tukey's test (pairwise comparisons of the means) was performed for different days of the experiment (Table 5). Results of the test revealed that in the absence of fish kairomone, there was no significant effect of NP concentrations on the first day of the experiment. In the second day, 0.5 mg l^{-1} NP was found significantly different from the control, solvent control and the other NP concentrations. The significance was incerased with time, and in the third day of the experiment the effect of both 0.5 mg l^{-1} and 0.15 mg l^{-1} NP concentration was found significant on survival of the individuals (Table 5).

However, in the presence of fish kairomone, the significant effect of 0.5 mg l^{-1} NP was found even on the first day of the experiment, meaning that the fish

kairomone accelerated the effect of NP concentrations on the survival. The significant effect of the NP concentrations was increased with time, in the second and the third day of the experiment the effect of both 0.5 mg l^{-1} and 0.15 mg l^{-1} NP concentrations were found significant (Table 6).

Table 5: Results of Post-Hoc tests (Tukey's HSD) for the effect of the treatments (nonylphenol concentrations in the absence of fish kairomone) on survival of *D. magna*

	C	Sc	0.005 mgl^{-1}	0.01 mgl^{-1}	0.05 mgl^{-1}	0.15 mgl^{-1}
I. Day						
C						
Sc	1.000 ns					
0.005 mgl^{-1}	1.000 ns	1.000 ns				
0.01 mgl^{-1}	1.000 ns	1.000 ns	1.000 ns			
0.05 mgl^{-1}	1.000 ns	1.000 ns	1.000 ns	1.000 ns		
0.15 mgl^{-1}	1.000 ns	1.000 ns	1.000 ns	1.000 ns	1.000 ns	
0.5 mgl^{-1}	.095 ns	.095 ns	.095 ns	.095 ns	.095 ns	.095 ns
II. Day						
C						
Sc	1.000 ns					
0.005 mgl^{-1}	1.000 ns	1.000 ns				
0.01 mgl^{-1}	1.000 ns	1.000 ns	1.000 ns			
0.05 mgl^{-1}	1.000 ns	1.000 ns	1.000 ns	1.000 ns		
0.15 mgl^{-1}	1.000 ns	1.000 ns	1.000 ns	1.000 ns	1.000 ns	
0.5 mgl^{-1}	.000 ***	.000 ***	.000 ***	.000 ***	.000 ***	.000 ***
III. Day						
C						
Sc	1.000 ns					
0.005 mgl^{-1}	1.000 ns	1.000 ns				
0.01 mgl^{-1}	1.000 ns	1.000 ns	1.000 ns			
0.05 mgl^{-1}	1.000 ns	1.000 ns	1.000 ns	1.000 ns		
0.15 mgl^{-1}	.003 **	.003 **	.003 **	.003 **	.003 **	
0.5 mgl^{-1}	.000 ***	.000 ***	.000 ***	.000 ***	.000 ***	.000 ***

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns: non significant

Table 6: Results of Post-Hoc tests (Tukey's HSD) for the effect of the treatments (nonylphenol concentrations in the presence of fish kairomone) on survival of *D. magna*

	C	Sc	0.005 mgl^{-1}	0.01 mgl^{-1}	0.05 mgl^{-1}	0.15 mgl^{-1}
I. Day						
C						
Sc	1.000 ns					
0.005 mgl^{-1}	1.000 ns	1.000 ns				
0.01 mgl^{-1}	1.000 ns	1.000 ns	1.000 ns			
0.05 mgl^{-1}	.0995 ns	.995 ns	.995 ns	.995 ns		
0.15 mgl^{-1}	.058 ns	.058 ns	.058 ns	.058 ns	.195 ns	
0.5 mgl^{-1}	.000 ***	.000 ***	.000 ***	.000 ***	.000 ***	.000 ***
II.Day						
C						
Sc	.996 ns					
0.005 mgl^{-1}	1.000 ns	.996 ns				
0.01 mgl^{-1}	.996 ns	.885 ns	.996 ns			
0.05 mgl^{-1}	.559 ns	.000 ***	.559 ns	.885 ns		
0.15 mgl^{-1}	.000 ***	.000 ***	.000 ***	.000 ***	.006 **	
0.5 mgl^{-1}	.000 ***	.000 ***	.000 ***	.000 ***	.000 ***	.000 ***
III.Day						
C						
Sc	.987 ns					
0.005 mgl^{-1}	.991 ns	.512 ns				
0.01 mgl^{-1}	1.000 ns	1.000 ns	.738 ns			
0.05 mgl^{-1}	1.000 ns	.987 ns	.911 ns	1.000 ns		
0.15 mgl^{-1}	.081 ns	.308 ns	.007 **	.165 ns	.081 ns	
0.5 mgl^{-1}	.000 ***	.001 **	.000 ***	.000 ***	.000 ***	.000 ***

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns: non significant

For each different NP concentration, survival curves at various conditions (such as F + NP, F + Sc, F + No NP etc.) were shown in figures 7, 8, 9, 10, 11. The symbols used are explained as follows:

- NP: The individuals exposed to the only nonylphenol.
- F + NP: The individuals grown in the fish kairomone and exposed to nonylphenol.
- No F + No NP: The individuals exposed to the neither fish kairomone nor nonylphenol.
- F + No NP: The individuals grown in the only fish kairomone.
- No F + sc: The individuals exposed to the only solvent (ethanol).
- F + sc: The individuals grown in the fish kairomone and exposed to solvent (ethanol).

In the treatments of No fish kairomone with No NP and No fish kairomone with solvent control, death was not observed throughout the experiment, so that the ethanol concentration that was used to dissolve NP test solutions had no negative effect on the survival of the *D. magna* individuals. Also in the F + Sc and F + No NP treatments, death was not observed till the 48th (±2 h), and reached to 1.25 (± 0.5) at the end of the experiment.

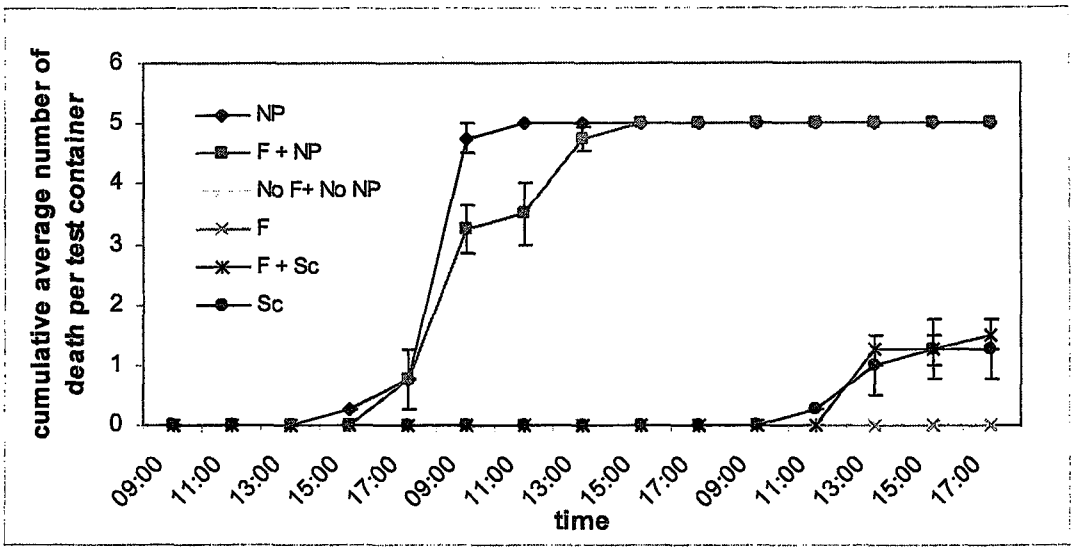

Figure 7: Cumulative average number of dead (±SE) *D. magna* per test container throughout the experiment at 0.5 mgl^{-1} NP concentration. NP: nonylphenol concentration, F: fish kairomone, Sc: solvent control.

As shown in Figure 7, at 0.5 mgl^{-1} NP concentration, death began in the 6th hour for both NP and NP with fish kairomone, increased with time and within the 24h (± 4h) reached maximum. Results given above are summarized in Table 7.

Table 7: Percentage of death per test container for the treatments at 0.5 mgl^{-1}NP.

Treatments	Percentage of death vs time		
	I. day	II. day	III. day
No F + No NP	0%	0%	0%
No F + sc	0%	0%	0%
F + sc	0%	0%	25%
F + No NP	0%	0%	30%
NP	95%	100%	100%
F + NP	65%	100%	100%

As shown in Table 7, in the first day of the experiment, death was observed only in the NP and NP with fish kairomone treatments. The percentage of death increased with time and reached to maximum at the second day of the experiment. Once more, in the F + No NP treatment death was observed in the third day with the percentage of 30, paralel to the F + Sc treatment. The percentage of death in this treatment was 25% at the end of the experiment.

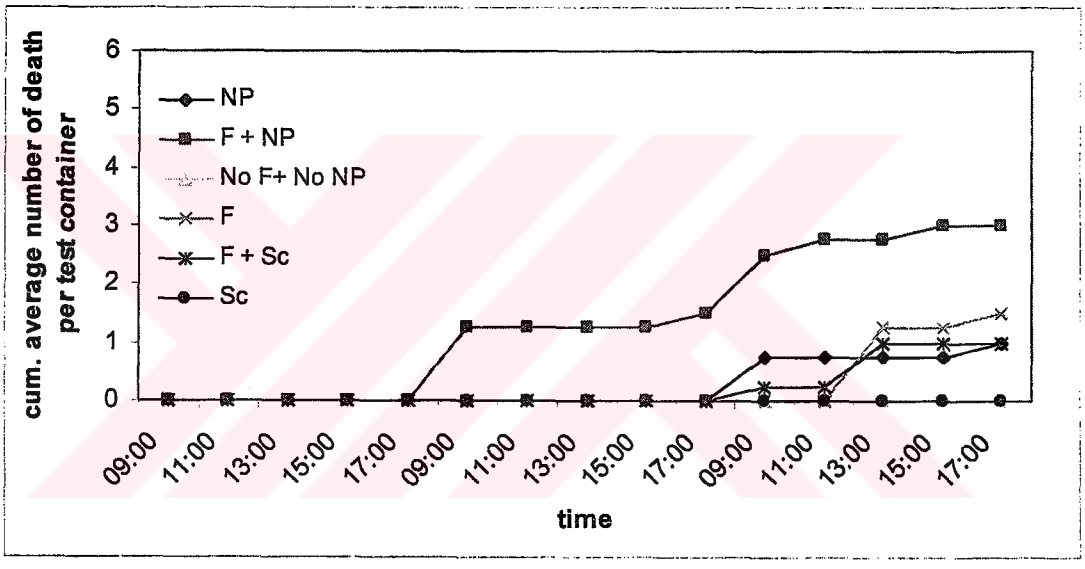

Figure 8: Cumulative average number of death *D. magna* per test container throughout the experiment at 0.15 mgl^{-1} NP concentration. NP: nonylphenol concentration, F: fish kairomone, Sc: solvent control.

At the 0.15 mgl^{-1} NP concentration, in the NP with fish kairomone treatment, death began in the 8th hour, increased through time and reached its maximum (3 individuals) at the end of the experiment. In the treatment of only NP, death was not observed until the 20th hour, increased through the time and reached to the average

number of 1 at the end of the third day. Results given above are summarized in the Table 8.

Table 8: Percentage of death per test container for the treatments at 0.15 mgl^{-1} NP.

Treatments	Percentage of death vs time		
	I. day	II. day	III. day
No F + No NP	0%	0%	0%
No F + sc	0%	0%	0%
F + sc	0%	0%	25%
NP	0%	0%	20%
F + No NP	0%	0%	30%
F + NP	0%	30%	60%

Table 8 shows that no death was observed until the third day in the treatments, except that the fish kairomone with NP treatment. In this treatment 30% of the individuals were death in the second day of the experiment. In the third day of the experiment, 60% of the individuals were death in fish kairomone with NP treatment, however, this percentage was only 20 in the treatment of only NP. In the same day, the percentage of death was 30 in the treatment of only fish kairomone and 25 in the treatment of fish kairomone with solvent control.

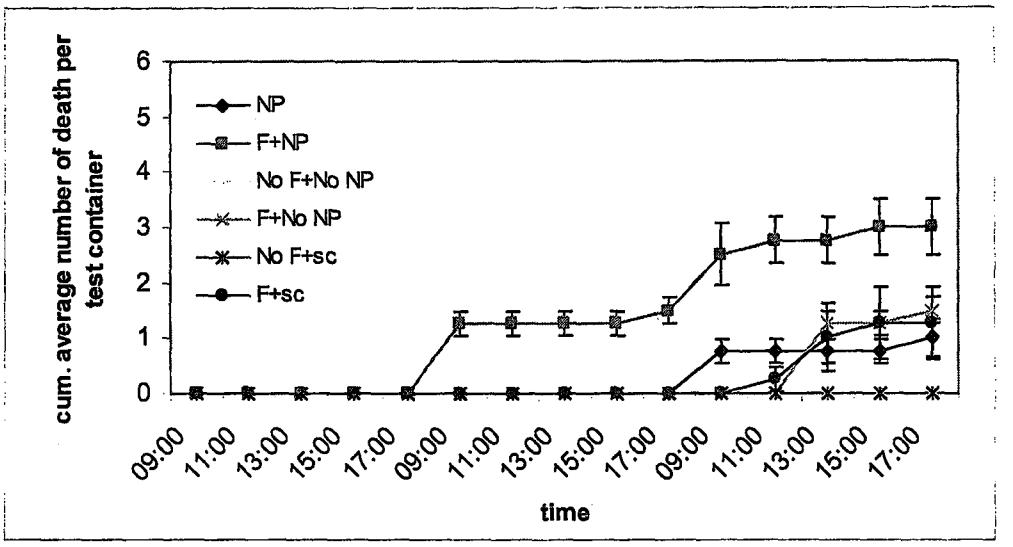

Figure 9: Cumulative average number of dead *Daphnia magna* per test container throughout the experiment at 0.05 mgl^{-1} NP concentration. NP: nonylphenol concentration, F: fish kairomone, Sc: solvent control.

Figure 9 shows that in the 0.05 mgl^{-1} NP concentration, death began in the 8th hour in the fish kairomone with the NP treatment, increased with time and reached the average number of 1 at the third day, end of the experiment. However, death was not observed in NP treatment throughout the experiment. Results given above are summarized in the following Table 9.

Table 9 shows that in the 0.05 mgl^{-1} NP concentration, similar to the 0.15 mgl^{-1} NP, death was not observed until the third day in all treatments except for the treatment of fish kairomone with NP. Death began in the second day in this treatment with the percentage of 10. In the third day, the highest percentage of death was observed in the fish kairomone with No NP treatment. The treatment of fish kairomone with solvent followed it, with the percentage of 25. In the treatment of fish kairomone

with NP, 20% of the individuals was found death at the end of the experiment.

Table 9: Percentage of death per test container for treatments at 0.05 mgl^{-1} NP.

Treatments	Percentage of death vs time		
	I. day	II. day	III. day
No F + No NP	0%	0%	0%
No F + sc	0%	0%	0%
F + sc	0%	0%	25%
NP	0%	0%	0%
F + No NP	0%	0%	30%
F + NP	0%	10%	20%

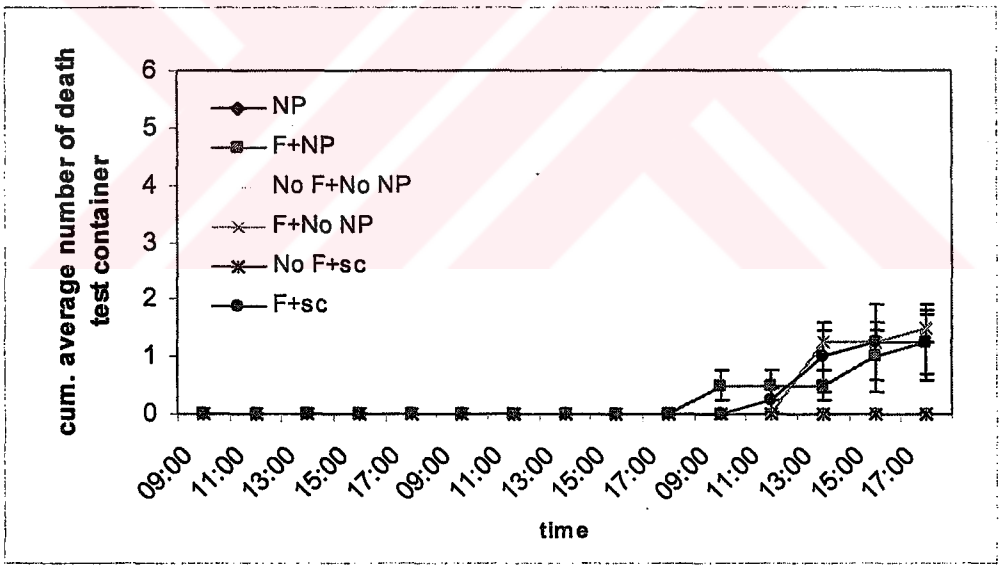

Figure 10: Cumulative average number of dead (±SE) *Daphnia magna* per test container through the experiment at 0.01 mgl^{-1} NP concentration. NP: nonylphenol concentration, F: fish kairomone, Sc: solvent control.

In the 0.01 mgl^{-1} NP concentartion, death began in the 32th hour in the treatment of fish kairomone with NP, reached 1.25 in the third day. Death was not observed in the NP treatment. Results given above are summarized in the following Table 10.

Table 10: Percentage of death per test container for treatments at 0.01 mgl^{-1}NP

Treatments	Percentage of death vs time		
	I. day	II. day	III. day
No F + No NP	0%	0%	0%
No F + sc	0%	0%	0%
F + sc	0%	0%	25%
NP	0%	0%	0%
F + No NP	0%	0%	30%
F + NP	0%	10%	20%

Table 10 showed same situation as found in the Table. There was no difference between the 0.05 and 0.01 mgl^{-1}NP concentrations.

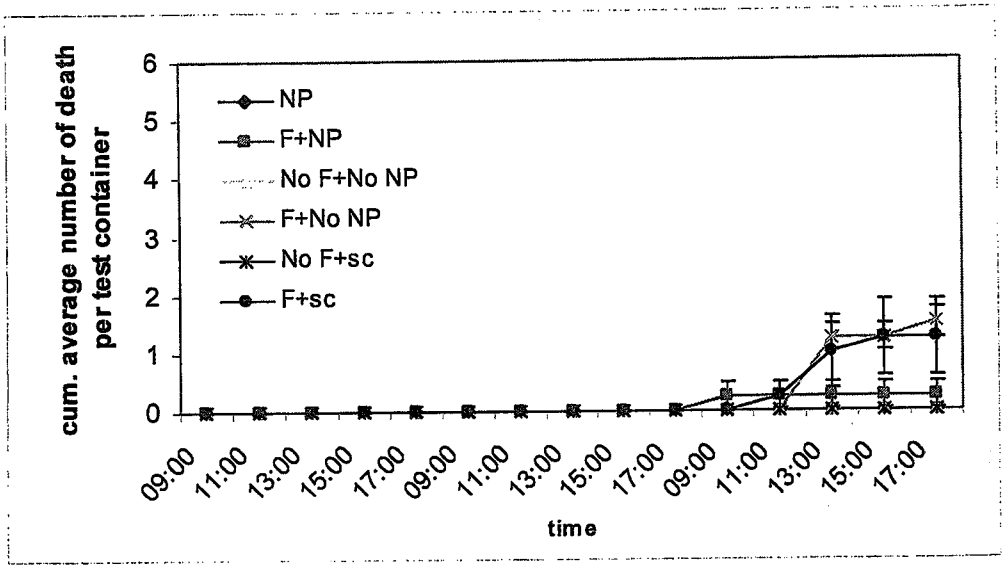

Figure 11: Cumulative average number of death (±SE) *Daphnia magna* per test container through the time at 0.005 mgl^{-1}NP concentration. NP: nonylphenol concentration, F: fish kairomone, Sc: solvent control.

Figure 11 shows that in the F + NP treatment, death was began in the 32 th hour with a value of 0.25 and remained same.

Table 11: Percentage of death per test container for treatments at 0.005 mgl^{-1}NP.

Treatments	Percentage of death vs time		
	I. day	II. day	III. day
No F + No NP	0%	0%	0%
No F + sc	0%	0%	0%
F + sc	0%	0%	25%
NP	0%	0%	0%
F + No NP	0%	0%	30%
F + NP	0%	0%	5%

Table 11 shows that there was no death until the third day, in all the treatments. In the third day, highest percentage of death was reached by the treatment of fish kairomone with No NP. Fish kairomone with solvent followed it with the percentage of 25, and finally the percentage of death in the treatment of fish kairomone with the NP was only 5.

3.2.Chronic toxicity experiment with varying food density

3.2.1. The effect of NP doses, fish kairomone and two levels of food concentration on survival of the individuals

To test the effects of different NP doses, fish kairomone and the two levels of food concentration repeated measures of ANOVA (GLM, SAS) was performed. The test revealed that the effect of NP, fish kairomone and their interaction were highly significant (Table 12). Moreover, the effect of time, time*NP, time*fish treatments were also found significant. However, the food effect was not significant (Table12).

Table 12: Results of Repeated measures of ANOVAs testing for the effect of the treatments (fish kairomone, NP concentrations, and food) on survival of *Daphnia magna*.

Treatment	DF	F	Significance level
NP	4	4.88	0.0014
Fish	1	39.70	<.0001
Food	1	3.43	0.0674
NP * Fish	4	3.89	0.006
NP * Food	4	0.15	0.9620
Fish * Food	1	0.27	0.6052
Error	84		
Time	5	34.08	<.0001
Time * NP	20	2.35	0.0009
Time * Fish	5	14.74	<.0001
Time * Food	5	1.89	0.0943
Time * NP * Fish	20	1.32	0.164
Time * NP * Food	20	0.15	1.000
Time * Fish * Food	5	0.24	0.9465
Error	420		

The effects of NP doses and fish kairomone on the survival of the individuals are shown in Figures 12, 13, and 14. The effect of food concentration was omitted in these figures since it was found that food concentration did not affect the survival of the individuals significantly (Table 12).

In generally, fish kairomone also had a negative effect on the survival of the individuals. Deaths began in the 9th day, increased with time and reached to the number of 2.25 individual per treatment at the end of the 21st day. The fish kairomone had a significant effect on the survival of the individuals. In the solvent control treatment, death was not observed until the day of 12, and reached to the average number of 0.5 at the end of the experiment. In the treatment which includes fish kairomone with solvent, death began in the 18th day, three day later at the end of the experiment reached to 1.25 individual per treatment.

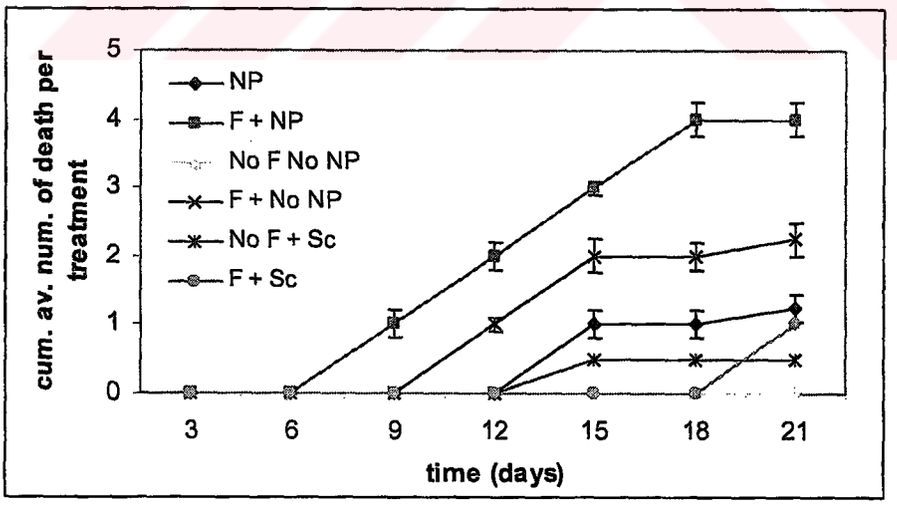

Figure 12: Cumulative average number (± SE) of dead *D. magna* per test container throughout the experiment at 0.01 mgl^{-1} NP concentration. NP: nonylphenol concentration, F: fish kairomone, Sc: solvent control.

At the 0.01 mgl^{-1} NP concentration, the highest cumulative average number of death was observed in the fish kairomone with NP treatment. The death began in the 6^{th} day, increased through time and reached to the average number of 4 individual per treatment in the 18^{th} day and remained the same till the end of the experiment. However, in the NP treatment death was not observed until the 12^{th} day and similarly increased with time and reached the number of 1.25 individual per treatment, at the end of the experiment. As seen in the Table 12, there was a significant difference between these two treatments.

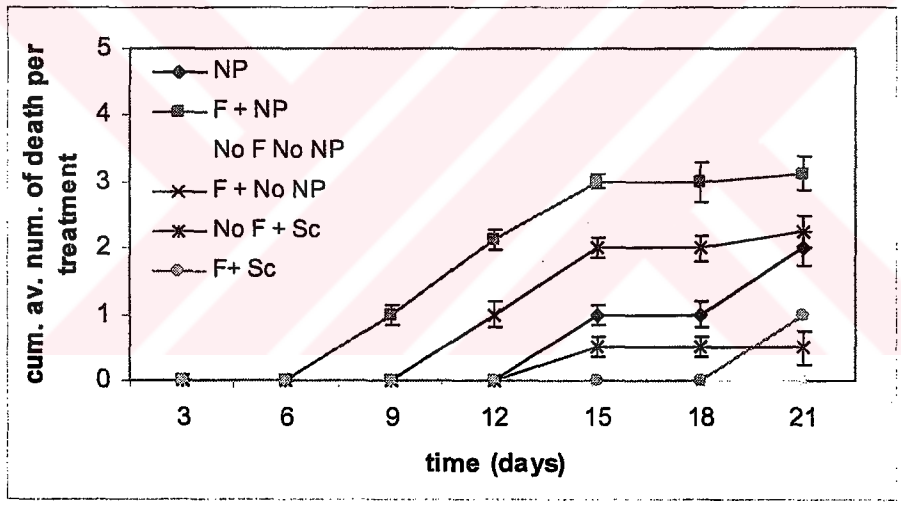

Figure 13: Cumulative average number (± SE) of dead *D. magna* per test container throughout the experiment at 0.005 mgl^{-1} NP concentration. NP: nonylphenol concentration, F: fish kairomone, Sc: solvent control.

Figure 13 shows that in the 0.005 mgl^{-1} NP concentration, similar to the 0.01 mgl^{-1} NP, the highest number of death was observed in the fish kairomone with NP treatment. The death began in the 6th day, increased thorugh time, and reached at its

maximum number 3,25 at the end of the experiment. But in the NP teatment, death began in the 12th day, and reached the average number of 2 at the end.

Moreover, in the treatments of F + NP and NP, the cumulative average number of death reduced in the 0.005 mgl^{-1} NP concentration compared to the 0.01 mgl^{-1} NP.

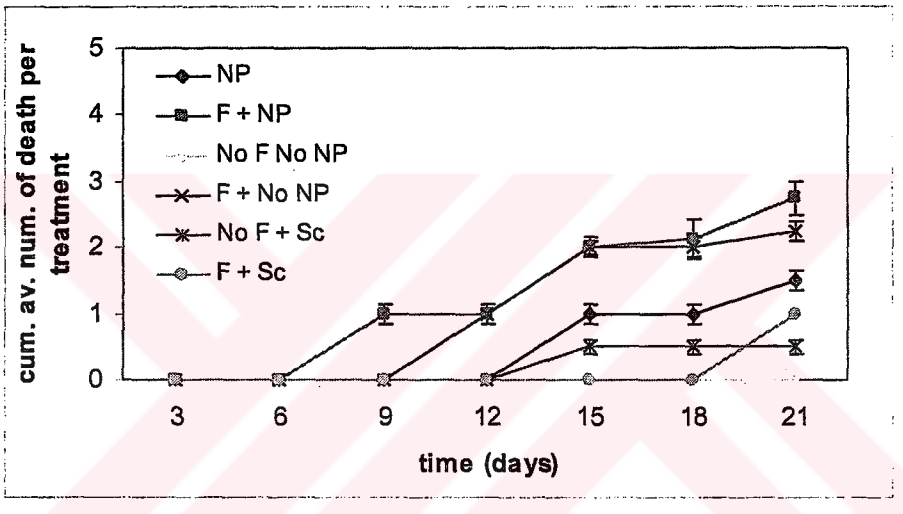

Figure 14: Cumulative average number (± SE) of dead *D. magna* per test container throughout the experiment at 0.001 mgl^{-1} NP concentration. NP: nonylphenol concentration, F: fish kairomone, Sc: solvent control.

The highest average number of death was again observed in the fish kairomone with the NP concentration at the 0.001 mgl^{-1} NP concentration. Death began in the 6^{th} day, increased through time and reached to its maximum (2.75) at the end of the experiment. Whereas, in the NP treatment, death was not observed until the 12^{th} day, and reached the number of 1.5 at the end of the 21^{st} day, at the end of

the experiment. The difference between the two treatments was also found in this NP concentration.

To have a better understanding of the effects of NP doses in the presence and absence of fish kairomone Tukey's test (pairwise comparisons of the means) was performed for different days of the experiment. Results of the test revealed that in both treatments the significant effect of NP doses began in the 21st day, at the end of the experiment and only at the 0.01 mgl^{-1} NP showed significant effect on the survival of the individuals (Table 13 and 14). Other concentrations of NP had no significant effect on the survival of *D. magna* both in the presence of fish kairomone and absence of it.

Table 13: Results of Post-Hoc tests (Tukey HSD) for the effect of the treatments (NP concentrations in the presence of fish kairomone) on survival of *D. magna*.

	C	Sc	0.001 mgl^{-1} NP	0.005 mgl^{-1} NP
Day 21				
C				
Sc	1.000 ns			
0.001 mgl^{-1} NP	.913 ns	.913 ns		
0.005 mgl^{-1} NP	.462 ns	.462 ns	.122 ns	
0.01 mgl^{-1} NP	.002 **	.002 **	.03 *	.03 *

** $P < 0.01$, * $P < 0.05$, ns: non significant.

Table 14: Results of Post-Hoc tests (Tukey HSD) for the effect of the treatments (NP concentrations in the absence of fish kairomone) on survival of *D. magna*.

	C	Sc	0.001 mgl^{-1} NP	0.005 mgl^{-1} NP
Day 21				
C				
Sc	1.000 ns			
0.001 mgl^{-1} NP	.934 ns	.934 ns		
0.005 mgl^{-1} NP	.584 ns	.584 ns	.124 ns	
0.01 mgl^{-1} NP	.002 **	.002 **	.03 *	.03 *

** $P < 0.01$, * $P < 0.05$, ns: non significant.

Results of Post-Hoc Tukey's tests also revealed that on 21th day (last day of the experiment) there was a significant effect of 0.01 mgl^{-1} NP on the survival of *D. magna* in the presence of fish kairomone (Table 13) and in the absence of fish kairomone (Table 14). Other concentrations of NP had no significant effect on survival of *D. magna* both in the present of fish kairomone, and absence of it.

3.2.2. The effect of NP doses, fish kairomones and two levels of food concentration on body length

3.2.2.1. x length:

Repeated measures of ANOVA (GLM, SAS) test revealed that the significant effect of NP concentrations, fish kairomone and food levels were significant on the x length of the individuals (Table 15). But interaction effects between them were not found to be significant (Table 15).

Table 15: Results of Repeated measures of ANOVAs testing for the effect of the treatments (fish kairomone, nonylphenol concentrations) on x lentgh of the *Daphnia magna*.

Treatment	DF	F	Significance level
NP	4	3.01	0.0271
Fish	1	26.36	< .0001
Food	1	18.75	< .0001
NP * Fish	4	.097	0.4317
NP * Food	4	1.22	0.3131
Fish * Food	1	0.72	0.4014
Error	48		
Time	5	188.13	< .0001
Time * NP	20	1.45	0.1018
Time * Fish	5	5.74	< .0001
Time * Food	5	5.72	< .0001
Time * NP * Fish	20	0.8	0.7179
Time * NP* Food	20	1.6	< .0001
Time * Fish * Food	5	1.04	0.7179

Tukey's test was performed to test the effects of each NP doses for the individuals were grown in fish-conditioned water and non-fish conditioned water separately. The result of the test revealed that in the absence of fish kairomone, only the highest NP concentration, 0.01 mgl^{-1} NP had a significant effect on the x length. The effects of other NP concentrations were found as non significant. The significant effect of 0.01 mgl^{-1} NP concentration was observed only the 6th day of the experiment (Table16).

Table 16: Results of Post-Hoc tests (Tukey HSD) for the effect of the treatments (NP concentrations in the absence of fish kairomone) on the length of *D. magna*.

	C	Sc	0.001 mgl^{-1} NP	0.005 mgl^{-1} NP
Day 6				
C				
Sc	.588 ns			
0.001 mgl^{-1} NP	1.000 ns	.588 ns		
0.005 mgl^{-1} NP	.998 ns	.863 ns	.998 ns	
0.01 mgl^{-1} NP	.04 *	.588 ns	.04 *	.122 ns

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns: non significant.

In the presence of kairomone, the 12th and the 21st days there were significant effects of NP concentrations. In the 12th day, 0.005 mgl^{-1} NP behaved like the control treatment and was different from the other NP concentrations. In the 21st day, the highest NP concentration, 0.01 mgl^{-1} NP was different from the control treatment, but not different from the other treatments.

Table 17: Results of Post-Hoc tests (Tukey HSD) for the effect of the treatments (NP concentrations in the presence of fish kairomone) on survival of *D. magna*.

	C	Sc	0.001 mgl^{-1} NP	0.005 mgl^{-1} NP
Day 12				
C				
Sc	.114 ns			
0.001 mgl^{-1} NP	.022 *	.885 ns		
0.005 mgl^{-1} NP	.903 ns	.02 *	.004 **	
0.01 mgl^{-1} NP	.21 ns	.998 ns	.747 ns	.042 *
Day 21				
C				
Sc	.994 ns			
0.001 mgl^{-1} NP	1.000 ns	.999 ns		
0.005 mgl^{-1} NP	.939 ns	.815 ns	.936 ns	
0.01 mgl^{-1} NP	.048 *	.084 ns	.14 ns	.051 ns

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns: non significant.

To understand the effect of NP doses, fish kairomone and food concentrations on the x length of the individuals, average x length was calculated separately for both treatment (in the absence and presence of fish kairomone) and also for both food concentration and compared. The results were visualized in the Figures of 15 and 16.

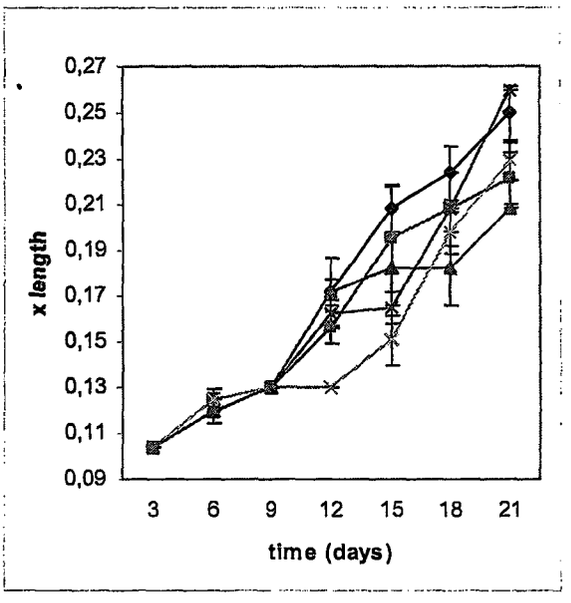

Figure 15 a

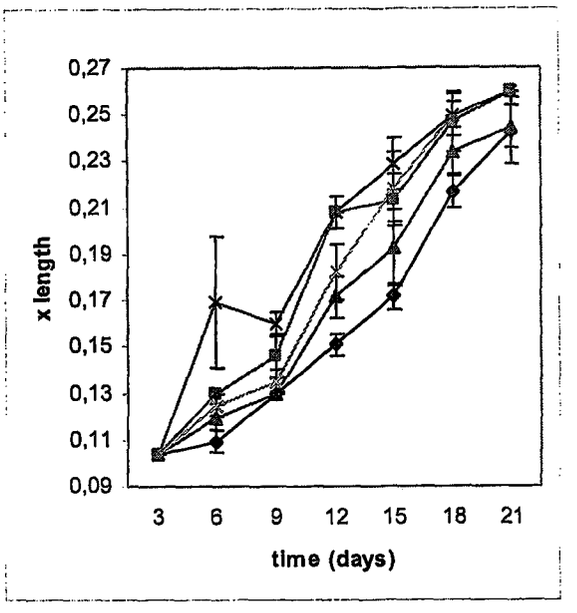

Figure 15 b

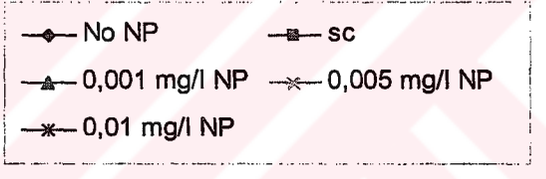

Figure 15: The average x length (± SE) of the individuals grown in different NP concentrations in the absence of fish kairomone a) in the low food, b) in the high food concentration. NP: nonylphenol concentrations, Sc: solvent control.

As the Figure 15 shows, there was a difference between the effect of different NP concentrations on the x length of the individuals that were fed with the low food and high food concentrations. In the low food condition, the individuals exposed to the 0.01 mgl^{-1} NP concentrations had the highest x length (0.25 mm) at the end of the experiment. The same was recorded for the high food condition. In the low food condition in No NP treatment x length increased to 0.25 mm. It was as high as the

0.01 mg l^{-1} NP treatment. However, in the high food level same treatment had the x length increased by 0.24 mm. The same was recorded at the 0.001 mgl^{-1} NP concentration in both the high and low food level. The x length of 0.005 mgl^{-1} NP and solvent control treatment also increased in the high food condition and reached 0.26

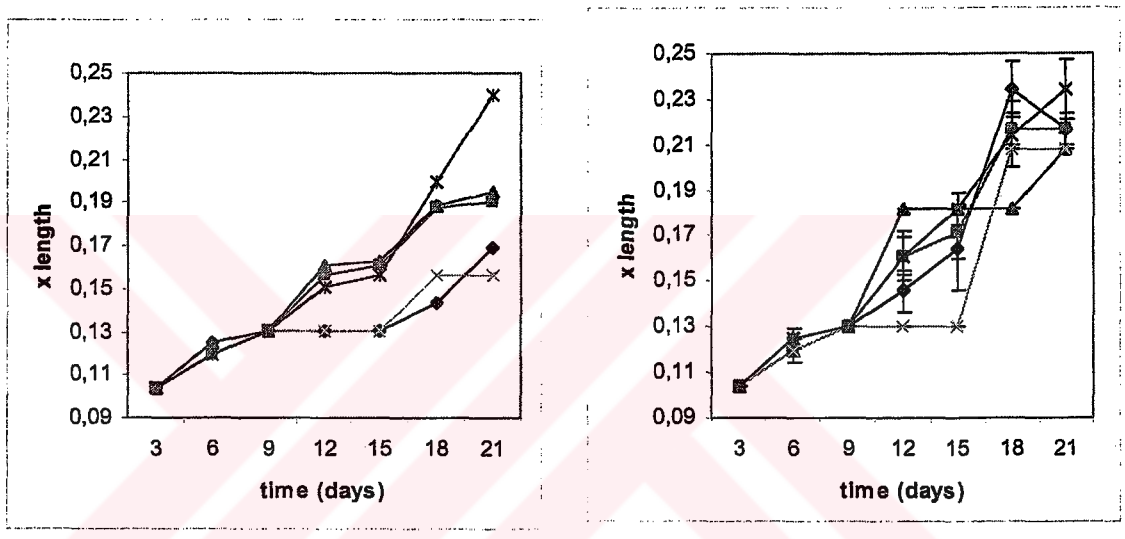

Figure 16 a

Figure 16 b

Figure 16: The average x length (± SE) of the individuals grown in fish-conditioned water a) in the low food, b) in the high food concentration. NP: nonylphenol concentration, Sc: solvent control.

Figure 16 shows that in the individuals were grown in fish-conditioned water, similar to the individuals that were grown in non-fish conditioned water, 0.01 mgl^{-1} NP had the highest x length in both low food and high food levels. In the low food condition, the treatment of No NP had the x length of 0.16 mm, whereas in the high food condition it increased to 0.22. At 0.005 mgl^{-1} NP concentration had the lowest x length at the end of the experiment in both food condition. The x length at 0.001 mgl^{-1} NP concentration and the solvent control teatment increased with the increasing food concentration and reached to 0.21 mm and 0.22 mm from 0.19 mm, respectively. As a summary, at 0.01 mgl^{-1} NP had the highest x length was observed in the both fish and food treatments.

3.2.2.2. y length:

Repeated meausres of ANOVA (GLM,SAS) revealed that fish kairomone and food concentration were higly significant on the y length of the *D. magna* (Table18). The NP levels and the interaction treatments were not significant on the y length.

Table 18: Results of repeated measures of ANOVAs for the effect of the treatments (fish kairomone, nonylphenol concentrations, food concentrations) on the y length of *Daphnia magna*.

Treatment	DF	F	Significance level
NP	4	1.22	0.3138
Fish	1	34.3	< .0001
Food	1	26.22	< .0001
NP * Fish	4	0.81	0.5255
NP * Food	4	0.89	0.4796
Fish * Food	1	2.37	0.1299
Error	49		
Time	5	220.2	< .0001
Time * NP	20	1.03	0.4276
Time * Fish	5	25.01	< .0001
Time * Food	5	10.48	< .0001
Time * NP * Fish	20	0.81	0.7025
Time * NP * Food	20	1.16	0.2885
Time * Fish * Food	5	1.12	0.3485
Error	245		

Since the effects of NP doses were insignificant, the data poll to discover the effects of fish kairomone and food levels (Figure 17 and Figure 18).

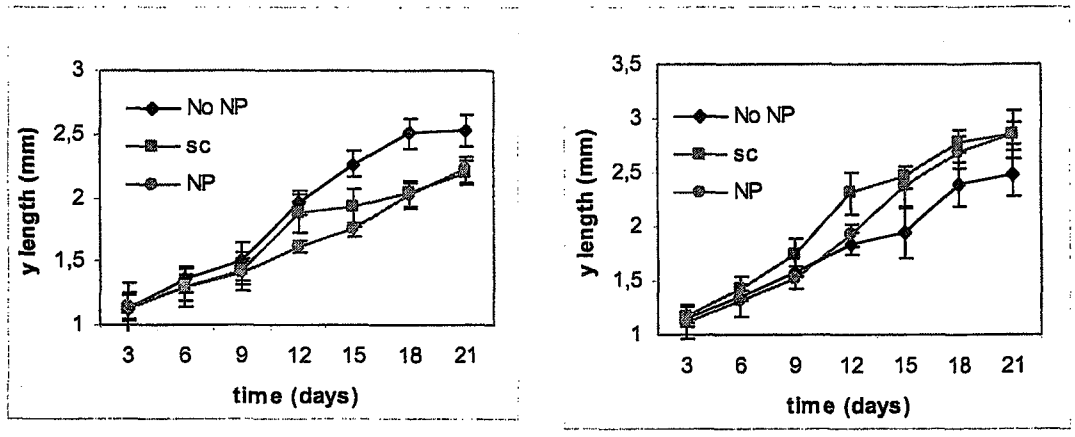

Figure 17 a

Figure 17 b

Figure 17: The average y length (± SD) of the individuals grown in different NP concentrations in the absence of fish kairomone a) in the low food concentration, b) in the high food concentration. NP: nonylphenol concentration, Sc: solvent control.

Figure 17 shows that there was a difference in the y length between the individuals grown in different food evels. There was an increase in the y length of the individuals in all treatments as the concentration of food increased. In the low food level, the individuals that in the No NP treatment had the highest y length, 2.5 mm, and it remained similar in the high food level. However, the y length of the individuals exposed to the solvent concentration and NP concentrations increased to 2.8 mm, from 2.25 mm.

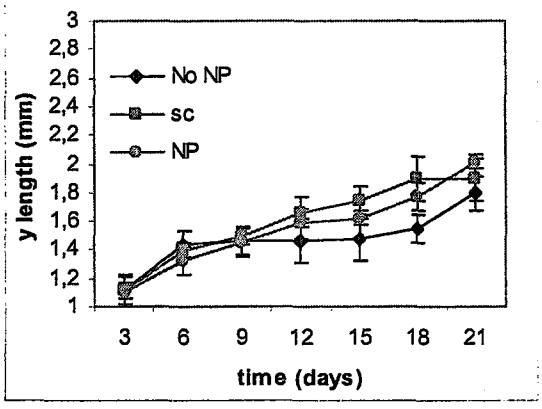

Figure 18 a

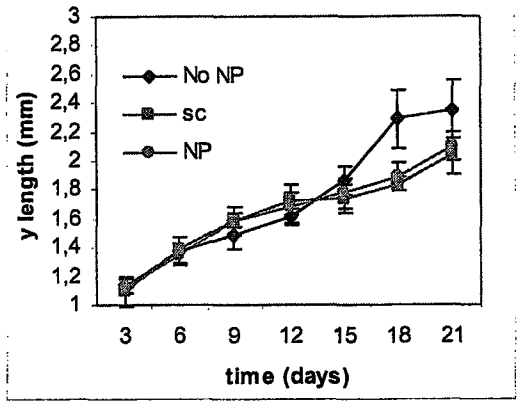

Figure 18 b

Figure 18: The average y length (± SD) of the individuals grown in fish-conditioned water a) in the low food concentration, b) in the high food concentration. NP: nonylphenol concentration, Sc: solvent control.

Figure 18 shows the difference in the y length of the individuals that were grown in high food and low food concentrations clearly. Similar to the individuals were reared in the fish conditioned water, these individuals also increased their y length with the increasing concentration of food. In the low food condition, No NP treatment had the lowest y length (1.75 mm), but in the high food condition, it increased to 2.3 mm which was the highest y length. The individuals grown in the solvent control treatment and in the NP treatment also increased their y value but the difference was not as much as observed in the No NP treatment. In the low food condition, the individuals grown in the solvent control treatment had 1.95 mm of their y length, in the high food condition it was increased to the value of 2 mm. The individuals that were exposed to the NP doses also increased their y length from the value of 2 mm to 2.15 mm which was not significant (Table18).

The individuals that were grown in fish-conditioned water had the lower y values than the individuals grown in non-fish conditioned water in all treatments. This result also manifested by the result of repeated measures of ANOVA (Table 18).

3.2.2.3.x/y length ratio:

Repeated measures of ANOVA (GLM, SAS) revealed that effect of NP concentrations was significant on the x/y length ratio of the individuals. The interactions of NP * Fish and Fish * Food were also found significant (Table 19). It was found that the other treatments were not significant.

Table 19: Results of Repeated measures of ANOVAs testing for the effect of the treatments (fish kairomone, NP concentrations, food concentrations) on the x/y ratio of *Daphnia magna*.

Treatment	DF	F	Significance level
NP	4	3.59	0.0126
Fish	1	0.16	0.6931
Food	1	1.66	0.2044
NP * Fish	4	4.03	0.0069
NP * Food	4	1.83	0.1398
Fish * Food	1	7.53	0.0086
Error	46		
Time	5	9.04	< .0001
Time * NP	20	1.53	0.3217
Time * Fish	5	1.64	0.1514
Time * Food	5	0.87	0.4999
Time * NP * Fish	20	0.83	0.6756
Time * Np * Food	20	0.5	0.9668
Time * Fish * Food	5	1.21	0.3036
Error	230		

To test the separate effects of different NP doses Tukey's test was performed. The test revealed that the NP concentrations had significant effects on the 12^{th} day and only the highest NP concentration (0.01 mgl^{-1} NP) was significantly different than control and the other NP concentrations.

Table: 20: Results of Post-Hoc tests (Tukey HSD) for the effect of the treatments (nonylphenol concentrations in the presence of fish kairomone) on x/y length ratio of *D. magna*.

	C	Sc	0.001 mgl^{-1} NP	0.005 mgl^{-1} NP
Day 12				
C				
Sc	.876 ns			
0.001 mgl^{-1} NP	.997 ns	.968 ns		
0.005 mgl^{-1} NP	.365 ns	.063 ns	.243 ns	
0.01 mgl^{-1} NP	.038 *	.313 ns	.123 ns	.000 ***

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns: non significant.

Figure 19 shows the effect of NP doses on the x/y ratio of the individuals visually.

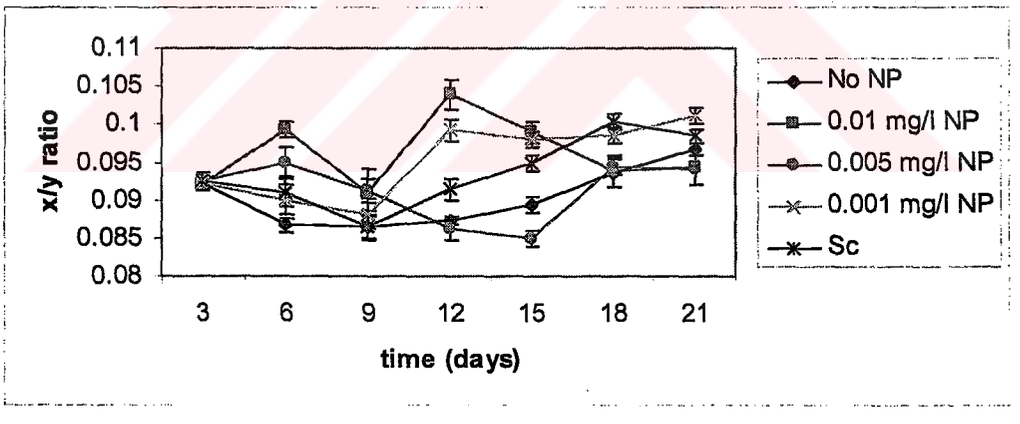

Figure 19: Average x/y ratio (± SE) of the individuals exposed to several NP concentrations. NP: nonylphenol concentration, Sc: solvent control.

In the No NP treatments, x/y ratio of the individuals was low in the beginning, and carried on to reduce till the mid-experiment. Then increased through the time till the end. Solvent control and 0.005 mgl^{-1} NP followed the same pattern

throughout the experiment. 0.01 mgl^{-1} NP concentration was different from the rest of the treatments in the 12th day, it has the highest x/y value. Tukey's test confirmed this result (Table 21).

3.2.3. The effects of NP doses, fish kairomones and two levels of food concentration on maturation time and mature size:

To see the effects of NP doses, two different food concentration and fish kairomone on the maturation time and mature size of the individuals MANOVA test was performed (Table 21).

Table 21: Results of MANOVAs for the effect of the treatments (fish kairomone, nonylphenol concentrations, food concentrations) on the maturation time and mature size of *Daphnia magna*.

Treatment	DF	F	Significance level
NP			
Maturation time	4	4.093	.005
Mature size	4	1.104	.362
Fish			
Maturation time	1	100.447	‹ .0001
Mature size	1	134.610	‹ .0001
Food			
Maturation time	1	.660	.420
Mature size	1	2.051	.157
NP * Fish			
Maturation time	4	3.574	.411
Mature size	4	4.049	.115
NP * Food			
Maturation time	4	.14	.967
Mature size	4	.527	.716
Fish * Food			
Maturation time	1	7.174	.009
Mature size	1	.022	.883
NP * Fish * Food			
Maturation time	4	.328	.858
Mature size	4	3.687	.009

3.2.3.1. maturation time

Results of MANOVA (GLM, SPSS) revealed that nonylphenol, fish kairomone were significant on the maturation time of the *D. magna* (Table 21). Especially effect fish kairomone was higly significant. Furthermore, food concentration was not significant when it was exposed only, but the test revealed that food concentration interaction with fish kairomone was significant on the maturation time of the animals. As the food concentration did not significantly affected the maturation time of the individuals, the data of high food and low food concentrations were pooled and Two-way ANOVA performed to test the effect of fish kairomone and the NP doses on the maturation time of the individuals (Table 22).

Table 22: Results of Two-way ANOVA for the effect of the treatments (NP concentrations in the presence and absence of fish kairomone) on maturation time of the *D. magna*.

Treatment	Significance level
NP	.001
Fish	.000
NP * Fish	.122

Results of Two-way ANOVA showed that both the effect of fish kairomone and the NP were highly significant on the maturation time of the individuals.

To see the separate effects of different NP doses on the maturation time of the individuals Tukey's test was performed (Table 23). Result of the test revealed that

both 0.005 mgl^{-1} NP and 0.01 mgl^{-1} NP significantly affected the maturation time of the individuals. But the effect of 0.001 mgl^{-1} NP was found non significant.

Table 23: Results of Tukey's test for the effects of NP concentrations on the maturation time of the *D. magna*.

	C	Sc	0.001 mgl^{-1} NP	0.005 mgl^{-1} NP
C				
Sc	1.000 ns			
0.001 mgl^{-1} NP	.350 ns	.277 ns		
0.005 mgl^{-1} NP	.032 *	.021 *	.045 *	
0.01 mgl^{-1} NP	.002 **	.001 **	.05 *	.028 *

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns: non significant.

The effect of NP doses and the fish kairomone on the maturation time of the individuals was shown in Figure 20.

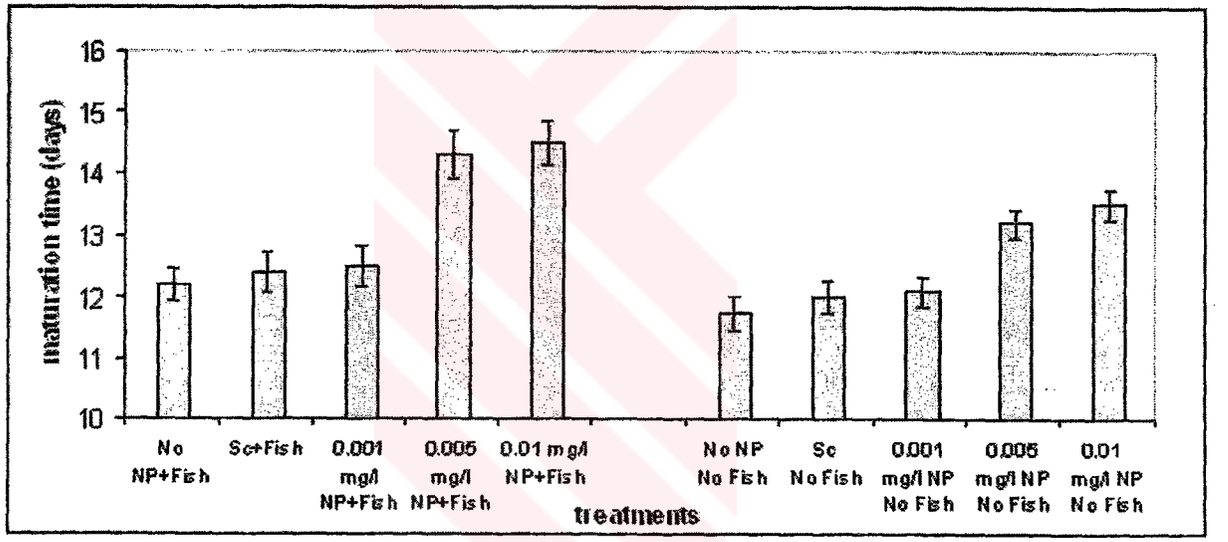

Figure 20: The effects of NP doses and the fish kairomone on the average maturation time (± SE) of the individuals.

NP: nonylphenol concentration, F: fish kairomone, Sc: solvent control

As Figure 20 shows, the individuals exposed to 0.01 mgl^{-1} NP concentration had the longest maturation time (14.5 and 13.5 day) respectively (both in the presence and the absence of the fish kairomone) and the individuals reared in the treatment of the No NP had the shortest maturation time with 12.2 days and 11.8 days in both treatments. In 0.005 mgl^{-1} NP treatment individuals that grown in fish-conditioned water had 14.25 days of maturation time, whereas this number reduced to 13.2 in the individuals that were grown in non-fish condition for the same NP concentration. In the absence of fish kairomone solvent control treatment and 0.001 mgl^{-1} NP treatment produced similar maturation time. However, the maturation time of the individuals in these treatments were reduced to 12 and 12.1 days, respectively in the absence of fish kairomone. As can be seen in the Figure 20, the individuals that were grown in fish-conditioned water needed to longer time for maturation as compared with the individuals that were grown in non-fish condition in all treatments.

3.2.3.2. mature size:

Results of MANOVA (GLM, SPSS) revealed that fish kairomone and its interaction with nonylphenol were significant on the mature size of the individuals (Table 21). Moreover, NP * Fish * Food interaction was also significant whereas neither nonylphenol nor food concentration were significant. Since the effect of food concentrations and the NP doses are found non significant on the mature size of the individuals, the data were pooled to see the effects of fish kairomone.

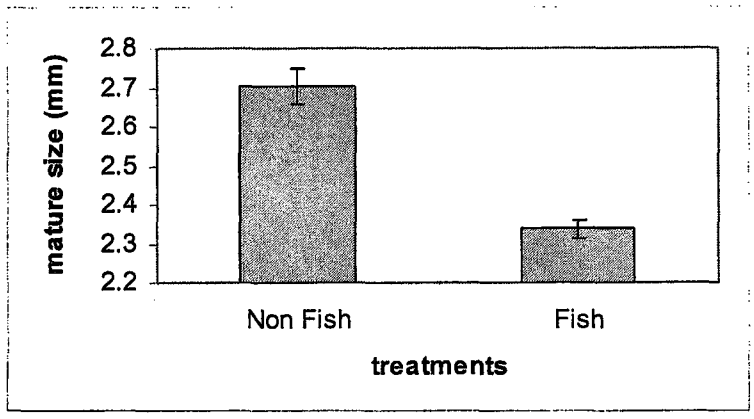

Figure 21: The average maturation size (± SE) of the individuals that were grown in fish kairomone and without fish kairomone. F: fish kairomone, Non fish: No fish kairomone.

Figure 21 shows that the individuals that were grown in non-fish conditioned water maturated when their length were 2.7 mm, however, the ones that grown in fish-conditioned water maturated when they were 0.35 mm smaller than the controltreatment. It is clearly seen that fish kairomone led to decreasing of the mature size of the individuals. The significance level of this effect can be seen in Table 21.

3.2.4.The effect of NP doses, fish kairomones and two levels of food concentration on the clutch size (egg number per individual):

To test the effect of fish kairomone, NP concentrations and two different food level on the egg number produced per individual, MANOVA was performed (Table 24).

Table 24: Results of MANOVAs testing for the effect of the treatments (fish kairomone, nonylphenol concentrations, food concentrations) on egg number per individual and of *Daphnia magna*.

Treatment	DF	F	Significance level
NP	4	0.838	.505
Fish	1	36.844	< .0001
Food	1	16.669	< .0001
NP * Fish	4	0.137	.968
NP * Food	4	0.163	.957
Fish * Food	1	2.255	.138
NP * Fish * Food	4	0.716	.584
Error	71		

Results of MANOVA revealed that fish kairomone and food concentration significantly affected the clutch size of *D. magna* (See Table 24). However, other treatments had not significantly effected the egg number. Since the effects of NP doses, we pooled the data and showed the effects of fish kairomone and food concentration on the egg number per individual *D. magna* in Figure 22.

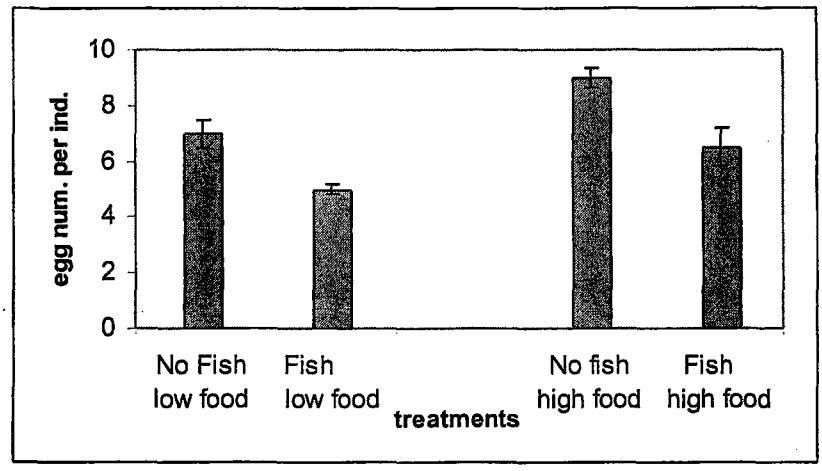

Figure 22: The effects of fish kairomone and food concentrations on the average (± SE) egg number produced per individual.

In the high food condition, the individuals that were grown in fish-conditioned water produced 6.5 egg per individual on average. However, this number was 9, for the individuals that were grown in non-fish conditioned water. In the low food condition, the individuals that were grown in fish-conditioned water produced only 5.2 egg per individual on average, whereas the ones that were grown in non-fish conditioned water produced 7 egg per individual on average. This results show that in the individuals that exposed to the fish kairomone produced less egg than the control ones in both high and low food condition.

CHAPTER 4

DISCUSSION

During the past two decades, the study of aquatic systems has received new impetus by the discovery of chemical messengers that are released by aquatic organisms (Havel, 1987; Tollrian and Harvell, 1998). Among other messengers, herbivorous zooplankton and predators of planktivorous organisms emit so-called kairomones (Lincoln et.al., 1982). Potential prey have the ability to detect these substances and phenotypically adjust several aspects of their life history, morphology and behaviour to increase fitness (Havel, 1987; Larsson and Dodson, 1993; Tollrian and Harvell, 1998). In *Daphnia*, it has been shown that the development of high helmets and neckteeth is an anti-predator response, because the protuberant structures have been shown to be effective in reducing vulnerability of a variety of *Daphnia* species to predation (Krueger and Dodson, 1981; Havel and Dodson, 1985; Mort, 1986; Parejko, 1990).

The morphological changes are, however, associated with a cost, which appears in some life history parameters in prey organism, for example *Daphnia*, the kairomones lower growth rate, reduce mature size, lengthen maturation time and reduce brood size (Havel and Dodson, 1987; Riessen and Sprules, 1990; Black and

Dodson, 1990; Spitze,1991; Hanazato and Dodson, 1992). Thus such changes in life history characteristics are considered to be negative. responses by *Daphnia* to the kairomone. Moreover, fish kairomone has also an ability to affect *Daphnia* indirectly, other than these direct effects. It has been claimed that the fish kairomone reduces the tolerance to environmental stress such as high water temperature, food shortage, low oxygen concentration and toxic chemicals (Hanazato, 1991b, c; Hanazato and Dodson, 1995).

The extent to which *Daphnia* modify their life history traits in order to minimize the predation threat and increase their fitness also dependent on food availability. The food level also affects several life history traits. At a low food level, daphnids show a delay in reproduction, a larger size and higher age at maturity, and a larger size of newborns, compared to at a high food level. An increase in size is known to provide a higher resistance against starvation (Threlkeld, 1976). If food is scarce, the physiological mortality is high (Lampert, 1978) and the intrinsic rate of population increase is lowered as compared to high food levels.

Toxic chemicals like nonylphenol have also been shown to affect *Daphnia*; the 24 and 48h EC_{50} values of nonylphenol, NP, to *D. magna*, based on immobilization, were determined as 0.30 (0.26-0.35) and 0.19 (0.17-0.21) mg/l (Comber et al., 1993) and in the study of Shurin and Dodson (1997), prenatal exposure to nonylphenol caused a non-lethal but disabling abnormality in *Daphnia* developing from embryos into juveniles.

Today, many lakes were contaminated with the anthropogenic toxic chemicals and although there are several studies investigating the effects of these chemicals on aquatic organisms, the interaction of the effects of natural stressors (fish kairomone, starvation, low oxygen concentration) and toxic chemicals is often neglected by researchers.

In this study, the effects of NP concentrations, fish kairomone and two different food level on the survival, morphology and life history characteristics of *D. magna* were investigated.

Two bleak (*Alburnus alburnus*) were incubated in 10 liters of aged and dechlorinated tapwater to prepare fish kairomone. This condition is in line with the literature (Brewer et.al., 1993; Loose and Dawidowicz, 1994). Bleak is a planktivorous fish, which feed on zooplankton, especially on *Daphnia*.

Results of the acute toxicity experiment showed that NP, fish kairomone and the interaction of these two factors significantly affected the survival of the individuals (Table 4). Both NP and fish kairomone led to increase of the cumulative average number of death in containers. Moreover, fish kairomone and NP effects appeared to be synergistic. They together developed strronger effect than that of either treatment.

The 24 and 48h LC_{50} values of NP for the individuals that were grown in fish condition were determined as 0.394 mg/l (0.31-0.51) and 0.149 mg/l (0.111-0.243), respectively. However, in the absence of fish kairomone, for the concentrations of 0.15, 0.05, 0.01 and 0.005 mg/l NP percentage of the dead *D. magna* was found 0%.

LC_{50} values for the individuals that were reared in the absence of fish kairomone could not be calculated due to the distribution of high survival rate (number of death; 0, 0, 0, 0, respectively).

These results showed that in the short run fish kairomone did not affect the survival of the individuals, but through the time the individuals that were exposed to fish kairomone in their early life stages, are more sensitive to NP. Results of the chronic toxicity experiment also confirmed this result. The individuals that were grown in fish-conditioned water were found more sensitive to the NP concentrations.

The enhancing effects of the NP and fish kairomone on the survival of the individuals confirms the results observed by Hanazato and Dodson (1992). They similarly found that, *D. pulex* became more sensitive to carbaryl (a pesticide) in the Chaoborus-conditioned medium than in the control medium.

There can be two explanations for this synergistic effect: Firstly, development of protuberant structures and some other responses to the predator kairomone requires *Daphnia* to expand energy (Hanazato, 2001). This may reduce the amount of energy that *Daphnia* allocates to detoxifying the nonylphenol, therefore increasing the sensitivity of the animal to the toxicant. However, protuberant structures such as high helmets were not observed during the experiment, so this explanation seems unlikely. Then, the juvenile life stage of *Daphnia* is the most sensitive stage to toxic chemicals (Hanazato, 1991c). The fish kairomone increases maturation time of *Daphnia*, meaning that it prolongs the duration of the juvenile stage. This results in a longer period expose to the kairomone at the sensitive stage, thus increasing the overall sensitivity of *Daphnia* to the toxic chemical (Hanazato, 2001).

The results of the present study suggest that if the results of laboratory toxicity tests of nonylphenols (without fish kairomone) for *Daphnia* are generally to the same *Daphnia* species in predator-abundant lakes to evaluate the effects of the chemical on field *Daphnia* populations, the effects may be underestimated. The individuals living in predator-abundant lakes will be more sensitive to NP concentrations than the individuals used in laboratory toxicity tests in the absence of fish kairomone.

The effect of food concentration on survival of the individuals was not significant (Table 12). This result was rather unexpected. There could be two reasons for this result. Firstly, the food concentration which used to test the effect of low food on the individuals (0.075 mg C l^{-1}) was not a starvation dose for our individuals, though it was suggested in literature (Weber, 2001). Then, 21 day experimental period may not be enough time for this food concentration to affect the survival of the individuals.

Results of the Repeated measures of ANOVA revealed that fish kairomone significantly affected the x and y length of the *D. magna* (Table 15 and 18). The individuals that were grown in fish-conditioned water had smaller x and y values than the ones that were grown in the control water. However, the effect of fish kairomone on the x/y ratio of the individuals was found as non significant, meaning that the individuals that were exposed to the fish kairomone did not developed high helmets (Table 19). The development of high helmets in *Daphnia* has been shown as an anti-predator response, because the protuberant structures have been shown to be effective in reducing vulnerability of a variety of *Daphnia* species to predation

(Krueger and Dodson, 1981; Havel and Dodson, 1985; Mort, 1986; Parejko, 1990), and the development of the structures is induced by cues released from the potential predators (Havel, 1987; Dodson, 1989, Hanazato, 1995). But this effect of the fish kairomone usually observed in the neonates of the individuals not in the mothers.

Moreover, fish kairomone also decreased the mature size of the individuals (Table 19, Figure 21). The individuals that were exposed to the fish kairomone maturated at 2.7 mm, when they were 0.35 mm smaller than the individuals that were not exposed to fish-kairomone. This is in line with the results of the studies performed by other researchers (Vanni, 1987; Dodson, 1989; Leibold and Tessier, 1991; Tessier, et.al., 1992; Vonder Brink and Vanni, 1993; Hanazato, 1995) and can be explained that planktivorous fish perform size-selective predation, feeding on larger, more conspicuous prey (Brooks, 1968; Zaret and Kerfoot, 1975). This may cause a reduction in mean individual size and size at maturity in zooplankton populations (Brooks and Dodson, 1965; Lynch, 1979). To reach sexual maturity at smaller size in the presence of fish or fish kairomones is an advantage for *Daphnia*, which may allow individuals to produce offspring before they become vulnerable to fish predation.

Threlkeld (1976) found that low food concentration led to increase in x and y length of the individuals. He claimed that an increase in size in the individuals exposed to low food concentrations is known to provide a higher resistance against starvation and also found that it also led to increase the maturation time of the individuals. However, our results showed that the individuals that were fed with the low food concentration had significantly smaller body length compared with the ones

that were fed with the high food concentration and there was no effect of food concentrations on maturation time of the individuals. It may be concluded that in our experiment, daphnids choosed to decrease their size rather than to delay their maturation to cope with the starvation. Because, in a limited time, it will be advantageous to reproduce often, and so produce many juvenile in a stress condition such as in starvation. Similarly, Hanazato et.al. (2001) also found that food shortage can lead to reduced mature size. The effect of food concentration on x/y ratio of the individuals was found as non significant (Table 19).

NP concentrations also affected the x length of the individuals significantly in both fish-conditioned and non-fish conditioned treatments but only the highest NP concentration, that is 0.01 mgl^{-1} NP affected. It was found that 0.01 mgl^{-1} NP concentration led to increasie in the x length of the individuals. However, it did not affect the y length of the individuals. It is known that some insecticides such as carbamate and carbaryl also able to induce helmet development in the *Daphnia* (Hanazato, 1991a, 1992a; Hanazato and Dodson, 1993). This study showed that NP also able to induce high helmets in the *D. magna*, as similar to these two toxicants. The first report on insecticides inducing the formation of protuberant structures on *Daphnia* was published by Hanazato (1992), who demonstrated that *D. ambigua* formed high helmets in the juvenile stages (exactly the same response to the *Chaoborus* kairomone) when exposed to harmful concentrations of the insecticide carbaryl. Then he tested whether other pesticides have the same effect on *D. ambigua*, and found that some insecticides had the effect, whereas herbicides and a fungicide did not (1991d). The chemicals tested, two carbamate insecticides and four organophosphorus insecticides which stimulate the nervous system. Therefore,

Hanazato (1991d) hypothesized that some effect of the insecticides on the nervous system of the animals triggered the formation of the high helmet as an anti-predator strategy, which originally evolved as a response to chemicals released by predators. It is found that also endosulfan has the ability to induce high helmets (Barry, 1998). These results suggest that the response to insecticide exposure is a general phenomenon in cyclomorphic *Daphnia*. Nonylphenol is not an insecticide and its effect on the nervous system is not known but it increased the helmet size of the individuals in our study, it may be concluded that alkylphenols may have the ability to affect nervous system and in turn may have led to development of high helmets.

Our results have also showed that fish kairomone has significantly affected the maturation time of the *D. magna* (Table 21). *Daphnia* lengthened their maturation time when they were exposed to the fish kairomone at both high and low food conditions and in the all treatments. This is in line with the literature (Dodson, 1989; Hanazato, 1995) and it can be seen as a cost associated with the defense. NP concentrations also affected the maturation time of the individuals significantly and led to the increase in it. The findings in this study showed that the individuals that were exposed to the 0.01 mg l^{-1} NP maturated 14.5 days, which was 2.25 days later than that of control in the presence of fish kairomone (Figure 20). In the absence of it they maturated in 13.5 days which was still 1.5 days later than control. Hanazato and Dodson (1992) exposed *D. pulex* to different concentrations of carbaryl with or without the *Chaoborus* kairomone and they found that carbaryl also increased the maturation time both in the presence and absence of kairomone as similar to those in our results.

The food concentrations also affected the clutch sizes. The individuals that were fed with the low food concentrations produced less eggs than the ones were fed with high food concentration. This can be explained that in the low food condition daphnids may shift their energy to produce less but the larger sized neonates to provide a higher resistance against starvation.

Fish kairomone led to reducing in clutch size in *D. magna* (Figure 22). Conversely to our results, there were results which found an increase in clutch size in response to the fish kairomone in the literature (Dodson, 1989; Stibor, 1992; Weider and Pijanowska, 1993). It was shown that daphnids experiencing strong selective pressure by fish, either directly by exposure to fish or indirectly via exposure to their kairomones, shift their reproductive strategy to produce many, small neonates. These studiesexplained the life history shifts as positive responses of *Daphnia* because reduced mature size and offspring size produce small adults, which are less vulnerable to visually oriented predator fish, and reduced maturation time with increased brood size increase the population growth rate to compensate for population reduction by predation. However, our experiment shows that the response of *Daphnia* to fish kairomones is similar to the response of to food deficiency. The fish kairomones might alter *Daphnia* behaviour, morphology and some other characteristics, and force the animals to lose more energy or gain less energy resulting in negative responses. The decreased energy gain may be a probable factor inducing the responses. The results of this study were in accordance with Hanazato et. al. (2001).

CHAPTER 5

CONCLUSION

In the chronic toxicity experiment, fish kairomone and NP significantly decreased the survival of the test organisms compared to the effects of either treatment. The effect of fish kairomone was stronger than the NP concentrations; however, at the highest NP concentration the effect was pronounced. Presence of fish kairomone and NP together significantly increased the maturation time whereas fish kairomone itself significantly decreased the maturation size. The high food level and the NP concentrations increased the helmet size, and the former also increased clutch size.

The present study showed that, upon exposure to the fish kairomone, based on the survival sensitivity to NP increases and life history characteristics change as follows: delayed maturation time (slow growth), decreased mature size, increased helmet size, and decreased egg number per individual. In conclusion, *Daphnia* are expected to be more vulnerable in eutrophic lakes, which receiwed sewage effluent due to very high planktivorous fish stock and likely presence of NP.

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APPENDIX A

PREPARATION OF PROTEOSE PEPTONE MEDIUM

To 940 ml of glass-distilled water, add 1.0 g proteose peptone, 15.0 g agar, and the following stock solutions:

Table 25: Preparation of proteose peptone medium

ml	Stock solution	g/400 ml H_2O
10	$NaNO_3$	10.0
10	$CaCl_2 \cdot 2H_2O$	1.0
10	$MgSO_4 \cdot 7H_2O$	3.0
10	K_2HPO_4	3.0
10	KH_2PO_4	7.0
10	NaCl	1.0