UNIVERSITY OF NOVA GORICA
GRADUATE SCHOOL

Simon LUKANČIČ

EFFECTS OF ALLOCHTHONOUS SUBSTANCES ON TWO FRESHWATER INVERTEBRATES

Dissertation

Supervisor: assoc. prof. Anton Brancelj, PhD.

Nova Gorica, 2008

ABBREVIATIONS AND SYMBOLS

BCA	bicinchoninic acid
CDNB	1-chloro-2,4-dinitro-benzene
COD	chemical oxygen demand
DATT	definitive acute toxicity test
DSC	differential scanning calorimeter
DW	dry mass (i.e. dry weight)
EC_{50}	effective concentration
ETS	electron transport system
ETS/R	ratio between electron transport system activity and respiration
FTC-PSt3	flow-through oxygen minisensor
·OH	hydroxyl radical
LC_{50}	lethal concentration
N_{tot}	total nitrogen
$\cdot O_2^-$	superoxide
OXY-4	4-channel oxygen meter
PROT	total protein content
POF	polymer optical fiber
POS	polarographic oxygen sensor
R	respiration
SOD	superoxide dismutase
WRFTT	wide range finding toxicity test
WW	wet mass (i.e. wet weight)

Terms used with units

ETS activity = determined as µl of oxygen requirement per mg of animal mass per hour; metabolic potential of organisms.

Oxygen consumption = oxygen (in µl) used per animal (in total) per hour.

Specific oxygen consumption or **specific respiration rate** = mass specific level of respiration defined as µl of oxygen per mg of animal mass per hour; µl $O_2\,mg^{-1}h^{-1}$

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abstract

Pesticides that end up in freshwater have significant effects on biota. However, better understanding of how to measure these effects is needed. A novel technique which is useful in the ecological evaluation of pesticide polluted freshwater ecosystems is presented here. Examinations of actual oxygen consumption, i.e. respiration (R) of the crustaceans *Asellus aquaticus* L and *Gammarus fossarum* (Koch) are coupled with measurements of electron transport system activity (ETS). ETS/R ratio is a good predictor of stress caused by pesticides like copper sulfate, imidacloprid and atrazine. Furthermore, the effects of esfenvalerate exposure on *Gammarus fossarum* were examined with regards to heat production and the activity of the stress enzymes glutathione S-transferase and glutathione peroxidase. Nominal exposure concentrations higher than 1 mg l^{-1} of imidacloprid and atrazine show an effect on both tested species. Low concentrations of pesticide esfenvalerate (from 0.1 µg l^{-1}) show no effect on glutathione S-transferase activity while glutathione peroxidase activity was raised and calorimetric measurements confirmed that effect. Results of this two studies show that those three pesticides affect animal's metabolism by increasing the animal respiration and consequently heat production. Both method combinations could be used for better prediction of limit values in freshwater ecosystems.

1. INTRODUCTION

Ecotoxicology is concerned with the study of toxic effects, caused by natural or synthetic pollutants on (a) living organisms, (b) their communities and (c) ecosystems. Aquatic ecotoxicology is the study of the effects of manufactured chemicals and other substances of anthropogenic or natural source on aquatic animals. Methods used for toxicity determination are different and can range from simple laboratory toxicity tests to complex toxicity studies in environment. There are also different ecological data surveys, ways of calculation of toxic concentrations and statistical approaches (Cairns, 1983; Williams et al., 1985).

Ecotoxicological data about toxicity of substances found in nature are fundamental for establishing environmental risk assessments. Special emphasis in ecotoxicology should always be the expert responsibility on decision making processes for chemical pollution, especially when considering the limit values. This means that for definition of the limit value they should not consider only influences to one selected species, since single species tests can not predict responses at higher level. Determination of the limit values and potential maximal environmental burden should be done with developed parallel tests at higher level of organization. In order to get to the higher level, from the start also simple single species tests must be developed (Cairns, 1983; Lampert, 1984).

Environmental analyses in water hinterland (intensity and way of usage) give quantitative and qualitative indexes of water burdening but they lack information on influences on water organisms especially when the values of burdens are in low (i.e. sublethal) concentrations. Allochthonous compounds that are present in water can already give us insight to the present biotic conditions, but usually they are more complex than they appear (Cairns, 1983).

Substances that can be found in nature are from different origin and are transported by different vectors. Sometimes even by combination of them (Migliore and De Nicola Giudici, 1990). Allochthonous substances usually enter the biosphere from outside. They are always produced by industry and can be used in agriculture, transport, industry,… and end up in the environment. These substances can be washed by (storm) water to freshwater sources and rivers and also to underground water. They are usually diluted and partly decomposed to a variety of intermediate compounds - so called primary and secondary compounds. Intermediate compounds can have even more harmful influence on freshwater organisms and also to the whole ecosystem. Pesticides are manufactured in a way that they are necessarily toxic at least to a part of the biosphere, as a requirement of their function. This original compound is toxic and fate is usually known, but for all decomposed compounds the fate should be also investigated (Fernandez-Alba et al., 2002).

Animals used in eco-toxicological tests are selected according to the sensitivity of the species to the investigated substance. Furthermore these sensitive animals determine acceptable concentrations of chemicals that enter the natural systems. Information on the toxicity of compounds to macroinvertebrates can help in determining of maximum permissible levels of pollutants that should be allowed at discharges into freshwater ecosystems (Williams et al. 1985; Cairns and Niederlehner, 1987).

From all animals that could be candidates for ecological research, crustaceans are the most common choice, since they are among the most important elements of freshwater and marine food webs. They are present widely in freshwaters bodies and at least their sensitivity to the heavy metals should be determined (Migliore and De Nicola Giudici, 1990).

Daphnia magna is a preferred test organism for aquatic toxicology (Fernandez-Alba et al., 2002) but their parthenogenesis brings very similar responses of test animals due to low variability between animals. As a result the effects of compounds could be biased either to hyper- or hyposensibility of the test animals. The response to a specific influence of other test species that do not exhibit parthenogenesis can be much more diverse (McCahon and Pascoe, 1988).

Freshwater detritivores are widely distributed in rivers, lakes and ponds throughout Europe. Two species used in this assignment were selected as indicator species, the first one from order Isopoda *Asellus aquaticus* L. and second from order Amphipoda *Gammarus fossarum* (Koch). The first one is fairly resistant species that survives in severe conditions (Brown, 1976; Sket et al., 2003). The second species is less resistant and is found mostly in more turbulent water with higher oxygen concentrations.

A. aquaticus and *G. fossarum* generally occupy different zones in rivers. They both feed primarily on particulate organic matter, and have similar life history patterns, as they both reproduce from early spring to mid autumn. *G. fossarum* predominantly occurs in upper reaches and *A. aquaticus* in lover stretches of water courses. That kind of spatial separation is due to differences in physiological tolerances and competitive exclusion (Graça et al., 1994a). *A. aquaticus* has quicker life cycle and females produce greater amounts of eggs than *G. fossarum.* Both are suitable to be used in routine toxicity tests.

A. aquaticus is very sensitive species to heavy metals and can be used as a test organism in ecotoxicological test (McCahon and Pascoe, 1988; Migliore and De Nicola Giudici, 1990). *G. fossarum* also proved to be sensitive to the toxicant copper (McCahon and Pascoe, 1988; Blockwell et al., 1998).

Very important information is animal background, especially life history – the load of investigated substance that exists in source water at collecting site. Water and substrate can contain loads of heavy metals; animals that survive in that environment usually evolve tolerance to heavy metals, such tolerance can persist also to the next generation (Brown, 1976; Pascoe and Carroll, 2004).

The results of this study should provide information on the sublethal toxicity of copper sulfate, imidacloprid, atrazine and esfenvalerate in relation to the metabolism i.e. respiration. Sublethal concentrations used in experiments should be established on the basis of the toxicity test performed (at least ten fold lower concentrations according to LC_{50}).

Aim of the thesis

The main goal of this work was development of a technique that would be helpful for ecological evaluations of the conditions in freshwater ecosystem affected by human activities, especially agriculture. Main interest is based on measurement of physiological responses of some selected animals (indicators) in it. Such method could contribute to improvement of toxicity tests. The aim was to develop a single species method for environmental assessment studies on other animals that are known as a relatively susceptible species to the influences from their environment. Their reaction to a definite factor from their environment can be diverse in responses.

To achieve this goal three steps had to be done. At first "reference = standard conditions" had to be determined as "optimal conditions" for test animals during the resting. Knowledge of how acclimated animals react on laboratory environment is needed, especially on temperature and water quality. In the second step sublethal concentrations of four different chemicals, each one with different functional chemical group had to be determined (copper sulfate – inorganic compound, imidacloprid, atrazine and esfenvalerate – organic compounds). This can be done with the use of classical toxicity tests using different water concentrations of those substances with test animals. Determination of the effective concentration (EC_{50}) and the lethal concentration (LC_{50}) was done as the concentration at which 5 animals out of 10 were paralyzed (EC_{50}) or died (LC_{50}) (Clesceri et al., 1998). In the last step a toxic effects measurement of allochthonous substances was done. In particular the effect of substances on test animal metabolism was compared with reference conditions.

In first part of this work stress was estimated by respiration (R) measurement on a twin-flow microrespirometer and by measurement of electron transport system activity (ETS). Respiration measurement was used as a direct measurement of oxygen that gives information of the actual animal oxygen consumption. Measurements of the ETS activity give information on the maximum oxygen consumption that can be achieved by an organism (Musko et al., 1995).

The results should show if organisms are able to compensate bad environmental conditions with slightly higher respiration ratio. ETS (= potential respiration) should stay approximately the same at control and at treated groups of tested animals. We hypothesized that ETS activity in selected test animals will remain the same under all experimental conditions and only respiration rate, as result of stress, will change. We measured the ETS activity and respiration rate after exposure at different concentrations of selected allochthonous substances. To test this hypothesis main focus was on how a certain substance can change the physiology of the tested animal and how intensive those changes can be.

In second part of this work, another method was tested only on one test species and only on one pesticide – esfenvalerate. Stress after exposure was estimated by animal's heat production. Heat production was the main indicator of stress, followed by stress enzyme activity measurements. This work was accomplished in laboratory in Roskilde (Denmark) in limited time frame. That is why ETS/R stress measurement method and calorimetric methods have not jet been compared.

The hypothesis was that animal metabolism rate after exposure to esfenvalerate will raise and result will be seen as increased heat production measured in DSC microcalorimeter. According to the raise of heat emission, it is foreseen that also increased enzymatic activity will be observed in animal's tissue as a raise in stress enzyme production and activity of enzymes like glutathione peroxidase and glutathione S-transferase.

Theoretical background

1.1 Test animals

1.1.1 Selected animals

Crustaceans are frequently used as bioindicators in various aquatic systems as they are common and successful group of animals in marine, terrestrial and in freshwater ecosystems. The term **bioindicator** in a statistical sense is used to define group of organisms from the field which give information about the conditions/state in the environment. They can define the state by their presence or absence and by population dynamics. If some common species are absent in a certain geographical area, where they were present before, it means that this is indication of some severe changes. The term **biomonitor** is used to define an organism which can be used to establish variations in the bioavailability of contaminants by measuring accumulated concentrations in whole body or in specific tissues. Contaminants in the environment, which are at the moment of a bioassay not present any more, due to decomposition, can still be detected in animal tissues from that area. Considering that outside influences can be monitored and spotted after incidents (Plenet, 1995; Van Gestel and Brummelen, 1996).

Crustaceans are often used for comparative studies. Knowledge about adaptations in morphology and anatomy that separates species from each other is also relevant for identification of crustacean species. Some of their special features like respiration, circulation, excretion and osmotic regulation, reproduction and life history strategies are important for the interpretation of data from bioindicator studies using crustaceans for development of ecological methods (Rinderhagen et al., 2000).

Selected freshwater invertebrates as test animals were *Asellus aquaticus* L. and *Gammarus fossarum* (Koch). Adult males of both species were a choice due to higher repeatability and the simplicity of responses compared to females and juveniles. Females have different and more complicated stages in their life cycle. It is difficult to get females at the same stage without eggs or with similar amount of eggs. Juveniles are usually even more susceptible to pollutants, but it is harder to get them at the same development stage in-between two moults. Males can be picked out by similar size and color. In this way the whole method is more balanced (Migliore and De Nicola Giudici, 1987; Migliore and De Nicola Giudici, 1990).

Both species belong to the phylum Arthropoda, subphylum Crustacea, and class Malacostraca, subclass Eumalacostraca, superorder Peracarida. Furthermore genus *Asellus* belongs to order Isopoda, while genus *Gammarus* to order Amphipoda (Brusca and Brusca, 2003).

Order Isopoda

Most of 10.000 described species around the world are widely distributed in the sea. They are common inhabitants in nearly all environments also terrestrial, where the wood lice are the most successful terrestrial crustaceans (Brusca and Brusca, 2003).

Most of isopods are dorsoventrally flattened peracaridians. The name comes from similarity in morphology and orientation of their appendages. Some or all of pereopods of mature females bear oostergites that form a marsupium in which eggs are brooded. The abdominal appendages are five pairs of biramous pleopods and a pair of uropods. Thin permeable cuticle forms gills on those pleopods. Endopods of the second male pleopods are usually gonopods, which are used as intromittent organs (Sket et al., 2003; Ruppert et al., 2004).

Asellus aquaticus L

Water louse can be found in quite polluted freshwaters also in Slovenia. Troglomorphic species is *Asellus aquaticus cavernicolus* that can be found in cave system of the Pivka River (Cave Planinska jama). Other less numerous surface freshwater species is *Proasellus istrianus* and *Proasellus slovenicus* (Sket et al., 2003).

A. aquaticus is a species that has a wide geographical distribution. They are mostly found in ponds and slowly running waters (De Nicola Giudici et al., 1988). In Slovenia they can be found everywhere but in mountain streams. Main locomotion is walking; they are well adopted to live on slime ground. Mature male is bigger than female (up to 12 mm comparing to female up to 8 mm). In the nature coloration is usually drab with shades of gray, long antennae and legs are usually paler colored than main body. In the laboratory culture they are usually pale-brown (**see the Figure 1**). Chromatophores adapt body color to the background (Matoničkin, 1981; Sket et al., 2003).

Figure 1: Asellus aquaticus *L. from the Lake Cerkniško jezero.*

There are five marsupial stages in development of *A. aquaticus*. When juveniles are released they pass through two post-marsupial moults before seventh pair of thoracic appendages develops. The number of antennal segments and body length increase with age, so age estimation can be derived from these measurements. Isopods with their moults increase in width as well in length. Pereonal length and width are different at males and females (ratio at males 6:1; at females 3:1), this is closely correlated also with animal age (McCahon and Pascoe, 1988).
Growth is exponential from birth to sexual maturity and it is dependant of temperature as growth increases with increasing temperature. Sexual maturity is reached within 46-60 days at 15 °C, when average body length is 3.5 to 4.0 mm (Okland, 1978, cit. by McCahon and Pascoe, 1988).
Number of eggs produced by females depends on her body length. Females that are 4.0 mm long have on average of 21 eggs per brood, while females from 9 mm and over can produce 100 and more eggs. Brood development time is from 40 – 60 days at 5 – 10^{o}C. Another 150 – 200 day is required for them to produce next generation (Steel, 1961, cit. by McCahon and Pascoe, 1988).

Order Amphipoda

In order Amphipoda are roughly 8000 species. Most of them are marine, some are freshwater species and one family is terrestrial. The body tends to be laterally compressed. They have seven pairs of uniramous pereopods, first two pairs can be modified as chelae or subchelae. Coxal plates create protected ventral space to the thorax, which contains gills. This is branchial chamber and at females also marsupium. Biramous pleopods are used for swimming and also to create a current for ventilation through the branchial chamber. The urosome has biramous uropods that are heavily chitinised used for kicking or jumping. All those appendages are used for locomotion, which is normally swimming (Karaman and Pinkster, 1977; Brusca and Brusca, 2003; Ruppert et al., 2004).

The most frequent Amphipod species that can be found in Slovenia is stream scud (*Gammarus fossarum*). Related species, plain scud (*Gammarus pulex*), is very abundant in Western Europe, but was not yet recorded in Slovenia. Absence of *Gammarus pulex* in Slovenia allows *Gammarus fossarum* to colonize most of freshwater water bodies - from springs, ditches to bigger rivers. In Western Europe, the presence of more competitively successful species *Gammarus pulex*, *Gammarus fossarum* existence is limited only to springs (Sket et al., 2003).

Gammarus fossarum (Koch)

This species is also one of most abundant species in freshwater ecosystems in Slovenia and in some other parts of Europe, where competitive species (*G. pulex*) is absent or excluded (i.e. in springs). Wide geographical distribution and great maximum densities up to 10.000 / m^2 were reported. They are translucent slightly brown and gray (see the **Figure 2**); depending on food they can be also red, green or blue-green. They are benthic animals with very impulsive behavior; their periods of swimming are alternated with longer periods of crawling and burrowing. Males are up to 15 mm in length, while females are somewhat smaller. Precopulatory preguarding of female is very important in order to get fertilization done in-between the moults (Sket et al., 2003; Ruppert et al., 2004).

***Figure 2**:* Gammarus fossarum *(Koch) from the Iščica River.*

Juveniles that are released from the brood pouch from female posses 5 segments on the primary flagellum of each antenna. This number increases with growth progress. Age of cultured animals can be determined from the number of antennal segments and mean body length. The increase of temperature increases growth rate. Approximately 70 per cent of cultured juveniles survive and reach sexual maturity at 130 days at the temperature of 13 °C. Sexual maturity is reached at 14-16 antennal segments that are after 10^{th} moult when at male's genital papillae are visible and at females the oostegites are fully developed to form brood pouch (McCahon and Pascoe, 1988).

Female in nature has ability to produce 2-5 broods with a mean of 16 eggs (from 10 to 26). By increasing of temperature and excess of food at laboratory conditions it is possible to reduce time to sexual maturity. It is possible to culture animals through the all year round (McCahon and Pascoe, 1988).

Ecological importance of *G. fossarum* is shown by large covering of the globe and also use of amphipods in quality indexes. Temperature requirements of different subspecies of animals can be important. Optimum temperature for *G. fossarum* from reproductive aspect is very wide, from 4 to 19 °C. Optimal streams for *G. fossarum* stay cool in summer; their reproductive period extends from December to September next year. They can also breed in winter period with longer development time; more than 3 months and thus longer generation times (Pöckl, 1993).

1.1.2 Test animals in the laboratory

A test animal that is to be used in an experiment should be healthy and without unexpected influences. One among them is parasitism. The most common parasite found in *A. aquaticus* belongs to the genus *Acanthocephalus* (specifically *A. anguillae*). They use the crustaceans as their intermediate hosts and complete their life cycle (i.e. reproduction) in a fish. The parasite is transmitted to its final host when the isopod is eaten by a fish predator. Acanthocephalan parasites bring about severe phenotypic alterations to crustaceans. In particular, infected animals are melanized: their cuticle and legs become black. This is rather simple way to detect infection in a natural population. Alternatively, animals may harbor cestode parasites (Cezilly et al., 2000).

Safety precautions have to be taken when animals are collected from the field. When a test animal culture is established, animals need to be strong and vital without any environmental burden like parasites or other burdens like chemical substances. Infected animals should not be used in ecotoxicological tests as they are already under stress conditions. Animal metabolism is altered because of parasite activity and they can also change behavior including feeding activities. However parasites in lab culture can not spread like in natural ecosystem, since the final host is not present. When laboratory stock culture is established, there is only a little chance to be infected by internal parasites.

1.2 Metabolism

1.2.1 Basics of animal metabolism

Metabolism is sum of all changes i.e. chemical reactions in the organism. It includes food decomposition and absorption, osmoregulation, body development, anabolic and catabolic reactions and excretion (Peters, 1987).

Secondary production in aquatic ecosystem is addressed to all non-autotrophic organisms in lakes or rivers and varies with changing environmental conditions. Different species react different to those changes and their production is a result of many physiological processes. During metabolism certain amount of energy and/or matter is lost. That process is quite interesting and can be also used for estimation of production. A general scheme of secondary production can be expressed as:

Production = Assimilation – Respiration – Excretion (Schmidt -Nielsen, 1979)

Animals gain chemically bonded energy with oxidation of food; this process is referred to as their metabolism. Acquisition of energy can be an anaerobic (c. 10 % of heterotrophs) or aerobic (c. 90 % of heterotrophs) process. At aerobic metabolism in animals oxygen is used and oxygen is the one that determines their energy metabolism. Oxygen use binds all metabolic processes – so every activity that influences respiration also influences metabolism (Schmidt -Nielsen, 1979).

Metabolism as bio-chemical reaction depends on temperature. At ectoterm animals temperature is not controlled actively, but it is determined with the temperature of their environment. Basal metabolism is the lowest metabolism at given temperature, enabling organism to survive. It is determined for animals in standstill position, several hours after feeding and without any stressors; physical, chemical, physiological etc. (Schmidt-Nielsen, 1979; Peters, 1987). When metabolic rate is measured several different metabolic

levels can be recognized. It is important to know that states which can affect measurements (Randall et al., 2000). Total metabolism comprises basal metabolism plus energy costs of motion and increased activity (i.e. gathering food) and costs of digestion and reproduction. Knowledge of basal metabolism of animals is not enough at ecological researches, because motionless fasting animal does not have positive production rate. Aim of researches is to determine respiration during normal level of activity for the animal related to the given environmental conditions (Lampert, 1984).

Assimilation and biochemical transformation of ingested food is a phenomenon called specific dynamic action (SDA). All these contribute to energy expenditures (Philippova and Postnov, 1988). Other energy expenditures can be found in freshwater crustaceans. One of them is precopulatory mate guarding (PCMG) and another is moulting (Plaistow et al., 2003). An important energetic expenditure at females is egg production (Pöckl, 1993).

1.2.2 Mechanisms and consequences of stress

Homeostasis (also steady sate) is a complex equilibrium in all living organisms for maintaining their survival. This apparent harmony is constantly challenged by both externally and internally generated "stimuli". Adaptive responses are the ones that maintain successful survival as close to steady state as possible in a series of balancing and feedback activities reflecting an astounding array of biological and sociological behaviors (Selye, 1975).

The broad spectrum of stimuli capable of engaging this protective response is remarkable. Stress is a type of stimulation that is stronger and lasts for a longer duration, upsetting a typical perturbation response. The ability to exist in an ever-changing environment is a requirement for all life forms. This perturbation response is reflecting in an unexpected change in environmental conditions coming either from abiotic or biotic way and it is called stress. Abiotic stress is produced by natural factors such as: extreme temperatures, wind, drought and salinity. Biotic stress is beside animal interactions (especially inter- ad intra-specific competition, parasitism, predation) coming mostly from human activity resulting in changed environmental conditions i.e. from allochthonous substances. Abiotic stress is a naturally occurring factor that generally cannot be controlled by humans, but biotic stress can be controlled, especially when it is coming from human activities, like industry and agriculture. Plants and animals have the ability to adapt to abiotic stress over time (in evolution process), but sudden rise in the temperature demands from animals to take certain activities in order to survive (Selye, 1975).

One of important abiotic environmental factors regularly and predictably changed during the year is temperature. Poikilotherm organisms have evolved groups of enzymes, which activity is reversibly inactivated outside the conformed range (Yurista, 1999). Each individual consecutive group of enzymes in cascade has different temperature optimum. In this way in unfavorable conditions animals can "compensate" reduction in activity of one enzyme system with increase in activity of next one in line (Randall et al., 2000). In this case animal needs to quickly adapt, so i.e. respiration rate gets faster, consequently animal loses more energy, which has negative effect on growth and reproduction. Animal metabolic potential depends on concentration and type of enzymes; it is a measure of capability of an animal to survive in environment with certain temperature (Packard, 1971; Goss in Bunting, 1980).

Uncommonly high concentrations of natural substances also causes stress the same as toxicants introduced by men. Many different toxicants are the most common biotic stressors in nature. The effect of most of them is similar as the one from temperature described above. Enzyme systems need to adapt to environmental factors in perturbation responses as in long term stress responses (Selye, 1975). All these factors affect general metabolism with altering chemical reactions in an organism, especially respiration.

1.2.3 Respiration process - cell respiration

Respiration in general is constituted of oxygen uptake/consumption and release of carbon dioxide. This word applies as well whole organism as the processes in cells. Animal consume oxygen from the medium they live in and release carbon dioxide into it. Aquatic animals consume dissolved oxygen from water. Most of cells utilize oxygen for oxidation of foodstuffs. Carbon dioxide is synthesized and leaves body throughout the surface or the respiratory organs. The water formed in oxidation processes merely enters the general pool of water in the body and presents no special problems (Lampert, 1984).

Cell respiration at higher organisms is a process running in mitochondria (**Figure 3**) which are cell organelles of ellipsoid shape with double membrane. Outer membrane is permeable for parts as big as 10kD, while inner membrane is selectively permeable. Inner membrane is heavily folded (structures called cristae) and involves also enzymes of ETS; high energetic electron flow is provided to the final acceptor – oxygen (Voet and Voet, 2006).

Respiration involves glycolysis, the Krebs cycle, and electron transport. First two stages, the glycolysis and the Krebs cycle, are the catabolic pathways that decompose glucose and other organic compounds. Glycolysis occurs in cytosol, breaks glucose into two molecules of pyruvate. Pyruvate is transported through mitochondrion membrane by transport protein. The Krebs cycle takes place within mitochondrial matrix and completes the job by decomposing derivative of pyruvate (Acetyl coenzyme A) to the carbon dioxide. This is redox reaction in which dehydrogenase enzymes transfer electrons from substrates to NAD^+ to form NADH. The third stage of respiration is electron transport system (ETS) which is a chain that accepts electrons from the breakdown products of the first two stages, usually NADH. At the end of the chain there is molecular oxygen that is combined with two hydrogen ions to form water molecule (Campbell et al., 1999).

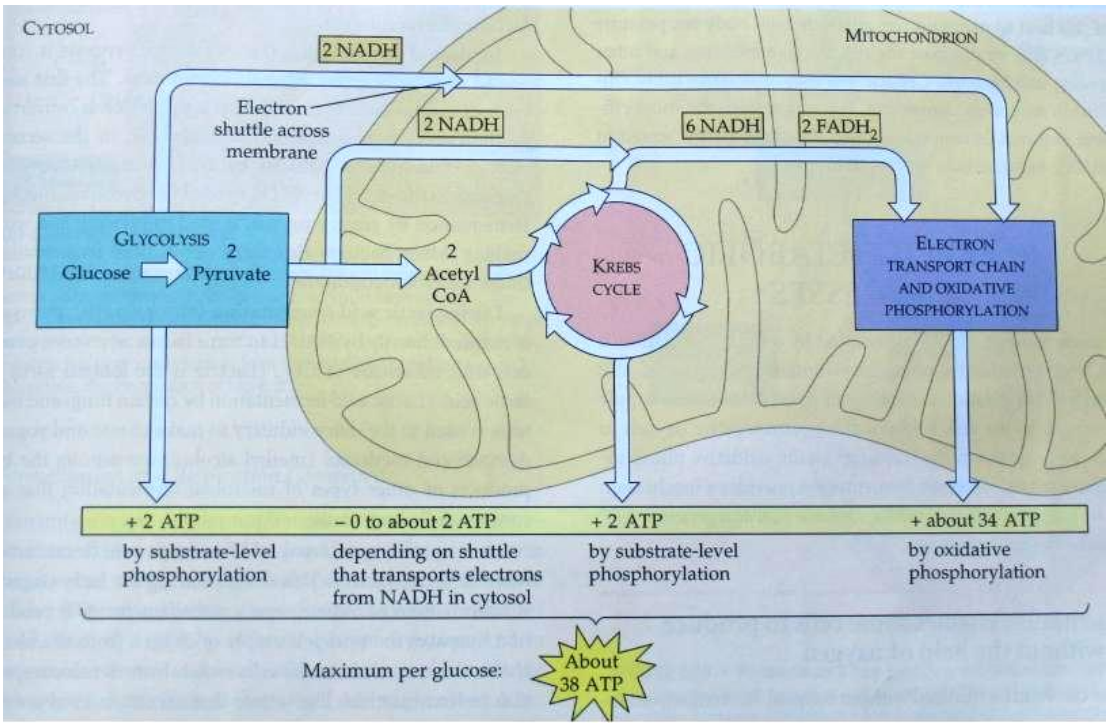

***Figure 3**: Flow-diagram of cellular respiration processes running inside and outside of the mitochondria and energy production (Campbell et al., 1999).*

Respiratory chain reaction is consisted of four complexes that are arranged by increasing redox potential (**Figure 4**). Complex I (NADH-coenzyme Q reductase) catalyses NADH oxidation with coenzyme Q. Complex II (succinate-coenzyme Q reductase) is cooperating at succinate oxidation. Electrons are carried to a coenzyme Q. By Complex III (coenzyme Q-cytochrome c reductase) are electrons transported from coenzyme Q to cytochrome c which transports electrons to Complex IV (cytochrome c oxidase). With its help oxygen reduction takes place (Voet and Voet, 2006).

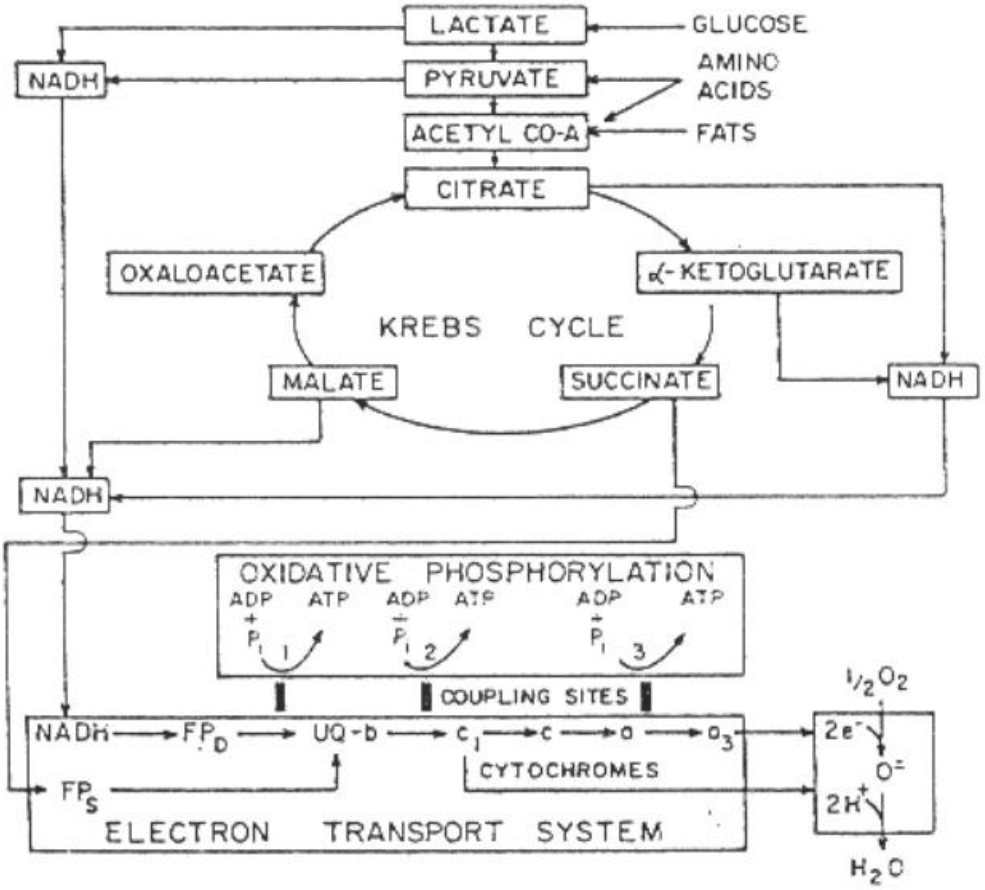

***Figure 4**: Krebs cycle, oxidative phosphorilation and ETS (Packard, 1971).*

1.2.4 Respiration mechanisms in Crustaceans

The locomotion of crustaceans mostly depends on the differences in habitat in which they are living. Benthic crustaceans usually walk or they combine swimming and walking, and different extremities can be used for both modes. Depending on size and shape of these extremities some of them functionally evolved also ventilation function. This ventilation enables respiration at crustaceans that occurs mostly via gills (Ruppert et al., 2004).

Modified parts of appendages of the thoracopods are usually with a thin cuticle and large surface area for gas exchange. Motion of these extremities, called “pleopod beats” sends a current of water with oxygen past the gills. In controlled experiments speed of pleopod beats can give quick information about the state of test animals. Any changes like increased ventilation movement of extremities, absence or reduction of animal locomotion can be a good predictor of environmental stress. Such observational method can give information on temperature preferences of animal (Dorit et al., 1991; Wijnhoven et al., 2003; Ruppert et al., 2004).

The respiration mechanisms make crustaceans susceptible to chemicals dissolved in water, due to easy intake through very thin cuticle (Ruppert et al., 2004).

1.3 Respiration measuring methods

1.3.1 Theoretical principles

Measurement of oxygen consumption has a long tradition in ecophysiological experiments. Under stabile and controlled laboratory conditions it reflects basic metabolism. Production of animals (i.e. secondary production) is a visible result of many physiological processes (= metabolism), which contribute to the change of animal's body mass; it is interesting to investigate closely the processes which contribute to metabolism and are triggered by the environmental conditions (Lampert, 1984).

Oxygen consumption measurement method in the experiments for water animals can be done either with closed bottle system or with open flow system with different sensors. Most frequently used in the past was **chemical determination** described by Winkler in 1888 (Clesceri et al., 1998). The reaction is based upon the oxidation of mangane hydroxide by the oxygen dissolved in water, resulting in tetravalent compound. In the second step dissolved oxygen is measured with titration. This is well known method and was modified by some authors in order to reduce the errors, and improve accuracy by better titration to adapt it to very small volumes of water (Lampert, 1984).

Much more advanced method is **open flow system** (also flow through). Compared to closed bottle system, measurements are continuous and the increased rates at beginning can be easily cut off. This increase is always present due to stress after handling and putting animal into small chamber. After a while animal calm state is attained and measurement is more accurate. Partial oxygen pressure in open flow system is constant and it can be calculated at any stage of the measurement. Excretory products are washed out, small volume of chamber allows animal to exchange gases continuously with the flow (Lampert, 1984).

1.3.2 Open flow system

For this system only tubes that are not permeable to oxygen must be used. There are several types and variations of the system:

(a) The first one is simple involving **siphon** that intakes water from Winkler type bottle and slowly passes water through the chamber with animal. Siphon must be long enough and tank large enough to assure a constant flow of water for some time. The end of the siphon is placed to another Winkler type oxygen analysis bottle. Oxygen concentration is determined chemically (Lampert, 1984).

(b) Systems with **polarographic oxygen sensors** (**Figure 5**) are improvement to former system.
Two systems are available for microrespirometer use. The first one is with one electrode and it does not provide continuous measurements. Water is slowly pumped from the reservoir through the test chamber to the electrode, where oxygen partial pressure is measured. After that measurement is interrupted since valve needs to be switched in order to measure oxygen concentration of the reference water (Lampert, 1984).

The second system was improved by Gnaiger and Forstner (1983) into method with twin-flow respirometer system with two polarographic oxygen sensors (POS), which are used for measurement of oxygen concentration alternatively before and after animal chamber. Two four-way valves with simultaneously switching increase the precision of the method because of very frequent recalibration. Measuring of oxygen is based on membrane covered polarographic oxygen sensor (POS). POS consists of pure platinum wire-sealed ring cathode and silver anode inside. Electrolyte solution (KCl) is fitted between both electrodes and covered with 25 µm Teflon-silicone double membrane that comes into contact with outside water. This is electrochemical device in which the steady-state current at working electrode (the cathode) is linearly proportional to the concentration of oxygen in contact with the external surface of the membrane. This system requires stirring in the measurement area; that potential pressure on the sensor does not drop. Measuring process also "consumes" oxygen during measurement, so it is not recommended for long term measurements in closed system. By switching it makes electrodes to alternate as calibration and measurement electrode. At the start they need time for calibration. Reiterate calibrations consume a lot of time for handling and calculations with a lot of averaging at the end is making data acquiring process more complicated.

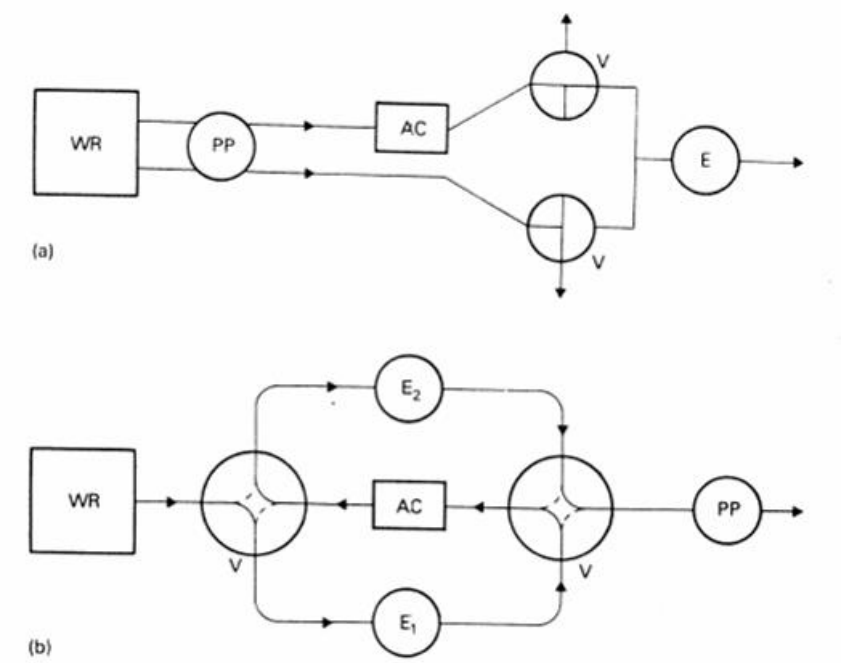

Figure 5*: Two systems for measuring oxygen consumption. a) System with one electrode; b) System with two electrodes – Twin-flow respirometer. WR – water reservoir, AC – animal chamber, E – electrode, PP – peristaltic pump, V – valve (Lampert, 1984).*

(c) Improved open system is introduction of fiber optic oxygen meter PreSens OXY-4; four channel oxygen meter with direct online oxygen measurements. Connections to flow-through fiber-optic oxygen minisensors are 2 mm polymer optical fibers (all PreSens GmbH).

Unlike electrode systems, optical sensors do not "consume" oxygen and no stirring is demanded. The signal is independent of changes in flow velocity which means that flow has no effect on the measured value. Modern oxygen consumption equipment produces data much faster, since values can be easily averaged and no complicated calculations are needed.

Change in respiration can be measured also indirectly with direct calorimetry. It is another way to measure metabolic changes, through measurement of change in heat production in test animal. Animal that is in stress produces more energy, that is spotted as change in heat emission measured in microcalorimeter (more details in Chapter 1.5).

1.3.3 Factors affecting the respiratory rate

There are some details that should be considered at measuring respiration and oxygen consumption. Body size and activity of the test animals are two important endogenous factors. Larger animals consume more oxygen (in absolute units) than smaller ones, but the respiration rate is often lower with the increase of the body mass. So if the respiration rate is compared on a body mass basis, it actually decreases with increasing size of the animals. Important changes in respiration rate are present also during development of an animal (ontogeny) (Lampert, 1984). Condition of an animal before and after moult cycle can change animal's respiration capability; at some stages animal can not even feed. Thus considerable errors can occur at measurements, if animals are taken from different stages or of different sizes and results are compared directly, without normalization.

Animal respiration should be measured at "normal" rates of activity. Additional stress can be shown in increased body activity which means higher energy expenditures, and higher respiration rate. Unnecessary influences (i.e. light) that could vary animal's activity should be reduced to minimum (Lampert, 1984).

There are also some exogenous factors that can affect respiratory rate and need to be taken into account. All factors need to be stable and present the term "controlled conditions".

Measurements should be taken at the same conditions. It is important that temperature and pH are not altered since they are the main factors that can change very quickly. Temperature has striking effects on many physiological processes, within the limits it can accelerate most processes. Very important are the animal's tolerance limits; generally a rise of 10 °C causes the rate of oxygen consumption to increase about two- or three- fold (for more see 1.4.2). Temperature during measurement should be stable (alterations within 0.1 °C) (Schmidt-Nielsen, 1979). Change in pH on the other hand can affect the fitness and increase the mortality of test animals (Hargeby, 1990).

Beside temperature and pH also effects of light, constant water current, constant oxygen concentration, no food during measurement and no crowding effect on animal in the chamber should be considered. All this is contributes to term "controlled conditions and all is important to assure that animals are not disturbed by any outside factor but stressor that we choose to test. It is highly recommended that measurements of oxygen consumption are performed in the dark (Lampert, 1984).

At oxygen reduction animals can retain the consumption of it to a critical pressure point; from that pressure point on a rapid decline in respiration is caused (Lampert, 1984). Measurements done at maximum level of oxygen saturation eliminate the effect.

Animals are usually not fed for 24 hour period before the experiment. When animals were fed before the experiment respiration rate usually increased compared to starved animals (Schmidt-Nielsen, 1979; Lampert, 1984). The excretion in animal chamber should be reduced too, while it can bring additional bacterial oxygen consumption due to decomposition of excrements.

A current is a factor that is important when studying animals found in running water like *G. fossarum*. Container size should also be kept small in order to prevent stagnation of water flow on the periphery of the animal chamber (Lampert, 1984). If only one animal is used in experiment crowding problem is also solved.

1.4 ETS method

1.4.1 Theoretical principles

Method was developed by Packard (1971) because of existence of many problems regarding direct measuring of respiration and oxygen consumption. First problem is stress on experimental organisms regarding putting them in small chambers that may cause greater consumption of oxygen than in normal conditions. Measuring *in situ* can also cause problem of the methods that are not sensitive enough. Packard (1971) evolved a method for evaluation of respiration that depends on biochemical determination of activity of the electron transport system (ETS) on the enzyme level. This complex is universal from bacteria to human and it is included in almost 90% of all the biochemical processes that run in biosphere at oxidation of organic matter. This method can be used for measurement of metabolic activity at aerobic and anaerobic organisms that possess respiratory chain enzymes. It is sensitive also at very low metabolic intensities (del Giorgio, 1992).

Packard (1971) established tetrazolium salt as an artificial acceptor of the electrons in measurement of electron transport system if suitable substrate is also present. ETS is in positive correlation with actual oxygen consumption at sea plankton. This activity is considered as metabolic potential. Different researches have altered and improved the method (Kenner and Ahmed, 1975a and 1975b; Owens and King, 1975; Christensen and Packard, 1979; G- Tóth et al., 1995a and 1995b).

ETS method is a chemical method used mainly to evaluate actual respiration potential of organisms. For transposing ETS/R ratio also physiological respiration measurement is advised. ETS/R ratio for specific group of organisms can be determined or it can be found in literature (Savenkoff et al., 1992). Quite big errors can occur if the constant values of ETS/R ratio are used for determination of intensity of respiration (Chapman et al., 1994). That is why determination of ratios is recommended every time for specific group of organisms.

There are two steps in the method. The first one is homogenization of sample and the second one is incubation of it in solution that consists of ETS substrate (NADH and NADPH) and solution with redundant 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrasolium chloride (hereafter INT) (G.-Tóth, 1993).

During a process of homogenization it is important that cells and also mitochondria's membranes are torn but at the same time enzyme activity should not be altered (Packard, 1971).

Main reaction is INT reduction; that is provided via oxidation of coenzyme Q- cytochromium b complex instead of reduction of natural electron acceptor – oxygen as normally happen in cells. The product of this reaction is non-soluble, red colored formazane which can be measured on spectrophotometer at wavelength of 490nm (G.-Tóth, 1993). ETS activity is measured on the site where speed of whole ETS activity is reduced, i.e. oxidation of coenzyme Q- cytochromium b complex (Packard, 1971). At this reaction ADP is not necessary because INT oxidative phosphorilation is separated from ETS (Borgman, 1977; G.-Tóth, 1993).

ETS activity can be explained as relative activity (A_{490nm}) or as electrochemical equivalent of oxygen derived from production of formazane. In mitochondrion two electrons and two protons are needed for transformation of ½ O_2 to water molecule. Two electrons are used for reduction of INT to formazane. That is why 2 µmol of formazane equals to 1 µmol of oxygen. Because 1 µmol of oxygen equals to 22.4 µl O_2 and 2 µmol of formazane ml^{-1} equals 31.8. We get ratio 1.42 A_{490nm} μl^{-1} O_2 (Kenner and Ahmed, 1975a).

Intensity of ETS rate depends mostly on metabolic potential of organisms but it depends also on conditions of analyses (entity of the sample, time of incubation, temperature, pH and substrate). Optimum conditions have to be accomplished for reaction process, so that enzymes can develop the highest activity (Voet and Voet, 2006). This is important when we determine metabolic potential correctly.

1.4.2 Optimal temperature and pH

Temperature affects the speed of both catalyzed and noncatalyzed reactions. Heating for 10^oC (Q_{10}) can accelerate speed of the enzyme reaction up to 2 - 4 times. Speed of reaction increases only to the critical temperature which is set right below the point, where protein starts to denaturate (Voet and Voet, 2006). Enzymes of ETS become thermally inactivated already at temperatures higher than 20 °C. This is specific for animals that are thermally acclimated to lower temperatures. Thus it is recommended that samples are incubated at lower temperatures (Kenner and Ahmed, 1975a).

It is recommended that samples are incubated at the same temperature and than with use of Arrhenius equation transposed to *in situ* temperature (Bamstedt, 1980) according to:

$$ETS_{in\ situ} = ETS_{ink.} * e^{Ea(1/Tinkubation - 1/Tinsitu)/R}$$

E_a (=µ) is activation energy, T is absolute temperature (in °K) and R is gas constant.

Optimal temperature for enzyme catalyzed reactions is not constant. It changes with biologic source of enzyme and with nature of analyses: ionic stage, pH, time of incubation (Packard, 1971).

Most of enzymes have specific range of pH in which they can be effective. For analyzing ETS in zooplankton they have optimal pH range from 8.4 to 8.5 (Owens and King, 1975; G.-Tóth, 1993). For different animal quite similar optimal pH values were obtained, for *Daphnia magna* they found optimal pH range from 7.8 to 8.1 (Borgmann, 1977).

At phytoplankton optimal pH obtained was 8.0 – 8.6 (Kenner and Ahmed, 1975a). The same values were found to be optimal also for the lake sediments (G.-Tóth, 1993), lake biofilms and fish embryos (G.-Tóth et al., 1995b).

1.4.3 Determination of metabolically active tissue

Determination of metabolically active tissue in animals compared to fresh body mass (i.e. wet weight) of animal is an important variable. A relation gives better idea of how ratios vary in animals at different sizes. Younger animals have different proportions compared to older and also small compared to bigger animals.

Proportion of metabolically active mass in *G. fossarum* can be estimated by using different variables and parameters, like chemical oxygen demand (COD), total nitrogen (N_{tot}), total phosphorus (P_{tot}) and mass of chitinous cuticle. Chemical analysis of total phosphorus is the most appropriate parameter for quantification of the metabolically active tissue in animal (Simčič and Brancelj, 2003). Alternative method is total protein content determination, which can be done at the end of experiments. Total nitrogen and total phosphorus method were tested as less appropriate in our case.

a) WW (wet mass) / DW (dry mass) ratio

Bigger animals have lower ratio of active tissue against inactive tissue. With increased size of the animals, a decrease in oxygen consumption per mass unit (inverse proportion) can be observed (Simčič and Brancelj, 1997). WW/DW ratio shows proportion of the water present in animal tissue. For better insight into animal's active tissue proportions total phosphorus determination can be used (Simčič and Brancelj, 2003).

b) Total phosphorus

From standard solutions the standard curve is calculated and results are acquired from it. Measured values of total phosphorus are calculated from the standard curve. Total phosphorus is afterwards recalculated from the mass of individual animal. Enzymatic measurement can not be performed on the same animal afterwards. Total phosphorus method is based on pre-made standard curve and all results are calculated from it. In this way accuracy and data on individual level are lost.

c) Total protein content

Proteins are the most complex and the most abundant organic molecules in the living cell, making up more than half the mass of a cell as measured by dry body mass. Their total mass compared to total animal mass can be used as a predictor of metabolically active tissues. Although the basic structure of all proteins is similar, a vast array of different proteins with diverse functions is found in biological systems (Randall et al., 2000).

Method of determining total protein content shows ratio between metabolically active and metabolically inactive tissue of test animals (like chitin). The method determines protein quantity and shows proportions of active tissue in animal. The test alone is quick and easy as it takes just a small part of the homogenate from animal. Unlike other methods, this one can use homogenate which is left from ETS method and does not require entire animal or uses just calculation of mass parameters. In that way additional individual information is accessed from each animal. This information can be used as a predictor of state of animals i.e. the moult stage.

BCA protein assay

This method is a detergent compatible formulation based on bicinchoninic acid (BCA) for colorimetric detection and quantification of total protein. The method combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (so called biuret reaction) with the highly sensitive and selective colorimetric detection of copper cation (Cu^{1+}) using a specific reagent containing bicinchoninic acid (Pierce, 2003).

The purple-colored reaction product of the assay is formed by the chelation of two molecules of BCA with copper ion. The water-soluble complex exhibits a strong absorbance at 562nm and is linear with increasing protein concentrations over broad working range (20 – 2000 µg ml^{-1}). The BCA method has undefined end-point; the final color continues to develop. That is why measurement of the color that evolved should be done in a short time after incubation (within 15 minutes). But following incubation the rate of continued color development is sufficiently slow to allow large number of samples to be assayed together (Pierce, 2003).

The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and thyrosine) are reported to be responsible for color formation with BCA. Studies with di-, tri- and tetra-peptides indicate that the extent of color formation is caused by more than the mere sum of individual color-producing functional groups. Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA) (Pierce, 2003).

1.5 Calorimetric measurements

There are two ways possible to measure metabolic losses and both are connected with energy budget. The first one is flow of energy and second flow of matter. Flow of energy can be measured by direct calorimetry. It is the method which measures direct emissions of energy by means of calorimeters.

There are three ways for measuring flow of matter (Lampert, 1984):
(a) measurement of oxygen consumption,
(b) measurement of CO_2 excretion,
(c) measurement of the ETS respiratory activity.

The experimental conditions for measuring of metabolic losses should be close to natural conditions in order to have animals at normal level of activity. If experiments are using different stressors, it is important that all other conditions are controlled and constant. But all the controlled conditions should be as close as possible to natural conditions.

In metabolism, energy is emitted as heat. Those losses of energy can be measured by direct calorimetry. For aquatic animals there are some problems with direct measurements; measurement cannot begin immediately. After animals are placed in the calorimeter the instrument is thermally disturbed. Re-equilibration takes time and in that time animal is using oxygen and excreting C (as CO_2). For best calorimetric measurement very small chambers with minimum quantity of water should be used. Improvement of this direct calorimeter is flow through system that includes low water flow. In this way animal would get aerated water and water with excreted CO_2 would slowly be removed (Lampert, 1984).

Heat capacity and changes in heat capacity as a function of temperature can be measured in Differential Scanning Calorimeter (DSC). DSC is able to precisely measure the apparent change in heat capacity involved in metabolism processes with a high sensitivity (Hart Scientific, 1991). Direct calorimetry is a good method in ecology for estimation of metabolism rates.

1.6 Toxicity tests

Toxicity test are desirable in water quality evaluations because chemical and physical tests alone are not sufficient to assess potential effects on aquatic biota. The reason is that the influence and effects of chemical interactions is complex and toxicity can not be determined from chemical tests alone. Different species of test organisms are not equally susceptible to the same toxic substance nor are they equally susceptible in different stages of their life cycle. Important matter is also previous exposure to toxicants; it may also change susceptibility of organisms and cause their adaptations. Organisms of the same species can respond differently to the same level of a toxicant from time to time, even when other test conditions are kept the same (Brown, 1976; Clesceri et al., 1998).

1.7 Toxic substances

1.7.1 Introduction

From the early beginning of agricultural practice man has fought against pests that affected crops, forests and other human managed ecosystems. At the start selection and different breeding and cultural practices were helping in reducing pest influence. But not for long, pest destruction and periodic crop failures sometimes also led to famine and widespread malnutrition, like the case from 1845 in Ireland with potato blight (Ware and Whitacre, 2004).

Technological advances, that helped to lessen crop losses, came in year 1883 as Bordeaux mixture (copper sulfate and hydrated lime) for controlling fungal diseases. But pest control originated already 1000 years BC with sulfur recognized as fumigant by Homer, it is still applied in larger amount than any other pesticides present nowadays (Gould, 1991).

Some plant delivered natural occurring substances like tobacco extracts as nicotine from 1960 on, were reported as effective as other synthetic pesticides. Next were pyrethrins from pyrethrum (*Chrysanthemum cinerariifolium* and *C. coccineum*) flower and rotenone from derris (*Derris* sp.) roots (Gould, 1991).

A real revolution in pest management came after second world war with the synthetic organochlorines and organophosphate insecticides, the herbicide 2,4-D, and halogenated hydrocarbon fumigants. Synthetically produced pesticides replaced inorganics and pesticides based on plant products in years from 1940 to 1980 were put to widespread use. Intensive use quickly showed environmental and human health risks, resulting in high economic costs. Pest control with those pesticides was very soon recognized also as not an ideal solution as the resistance evolution by many major pests appeared (Gould, 1991).

Organochlorines, organophosphates, methylcarbamates and pyrethroids were organic pesticides following the first generation. Most of them were quickly restricted due to resistant strains of pests that occurred as a result of their use. Pests limited use of pesticides to those that were still capable of bringing environmentally safe use compared to their effectiveness, including collateral damage. For example; arthropod resistance was first observed in Sweden in 1946, only seven years after DDT was introduced. By the year 1948 already 14 species of arthropods were reported to be resistant to DDT, carbamates, organophosphates, and cyclodiens or pyrethroid insecticides. Until year 1990 that number exceeded 500 species. Some major agricultural pest species developed resistance and growers responded to it by increasing the rate of application and after that with switching to another pesticide. Everything becomes ineffective since such a population of pests developed several different mechanisms of resistance against different pesticides (Gould, 1991).

In year 1995, the first genetically modified crops were introduced in pest - insect control war. These crops were able to express *Bacillus thuringensis* (*Bt*) δ-endotoxin as insect control factor (Tomizawa and Casida, 2003). With evolution of last generation of pesticides came neonicotinoid insecticides, which combine outstanding effectiveness with relatively low toxicity to non-target animals (Gould, 1991; Tomizawa and Casida, 2003).

In the last five decades people are aware of the risks of increasing use of pesticides to human health and to the environment. Traditional broad spectrum pesticide use is restricted in order to protect consumers, especially children, who are most susceptible to their effects. Pesticide producers replaced old generation organophosphorus and carbamate pesticides with an array of newly developed pesticides with selective action on only the target organisms. Though they are usually tested on variety of test organisms in laboratories, new evidences show that they still have many unexpected and unpredictable effects. Development of new measures of toxicity control is not a main concern of pesticide producers. Usual technique is determination of lethal concentration LC_{50} of the chemical that kills 50% of the population tested (Stark and Banks, 2001). This does not tell much of sublethal effects to animals, that is why it is important to explore new methods in this field of testing pesticide influences.

The United Nations estimates that nearly 99% of all pesticides used in agriculture miss their target; only 1-3 % actually reaches the crops. It is assumed that reminder ends up as contaminants in soil, air or water (Chiron et al., 2000; Malato et al., 2001; Krohn and Hellpointner, 2002).

Insecticides are among all pesticides used most frequently so they are greater threat to the environment than others, like herbicides and fungicides. In areas with intensive agriculture the peaks of pesticides in water can increase in four cases:

a) Pesticide treatment of crops,

b) Runoff water from equipment cleaning after application of pesticides,

c) Water coming from agricultural industry after cleaning crops or after postharvest treatment of crops,

d) Crop residues that can be disposed in fields (Chiron et al., 2000).

All these sources of pesticide use can end up like pollutants in running or standing water bodies as well as in groundwater.

In 1990ies there was increasing development of various methods for detection of pesticide concentrations in soil and in water (Sherma, 1993) and especially their degradation products (often hydrolysis products) are usually present at greater level in the environment than parent pesticide itself (Richardson and Ternes, 2005; Richardson, 2007).

The removal of those pollutants from the soil and water is not the only concern. The consequences of high peak pesticide concentrations on living organisms also need to be considered. Those peak concentrations influence soil and water body stability by changing species composition and thus indirectly the soil structure and water quality (Pantani et al., 1997).

The peak concentrations of chemicals are usually present for a short time and they usually do not kill populations of biota present in water body. But their influence can be disturbing in many ways for most of animals and plants present. The preliminary knowledge of the sensitivity of newly selected organisms to pure chemicals is very important. Standard toxicity tests usually concern only reference organism, like *Daphnia sp*., but it should be considered that other species can also contribute different reactions on chronic and acute exposures to tested chemicals at different concentrations (Pantani et al., 1997).

1.7.2 Heavy metals

For studying consequences of heavy metal pollution toxicity tests are usually used as a method for studying their effects. A wide concentration range used in toxicity tests were reported by several authors. Concentrations varied in scale from μg l^{-1} to mg l^{-1} for different elements (Migliore and De Nicola Giudici, 1990; Mullis et al., 1994; Brown, 1976 and 1977; Pantani et al., 1997; Boulanger and Nikolaidis, 2003; de Oliveira-Filho et al., 2004). That is why it is important to perform series of preliminary toxicity test on collected population of test animals, before actual measurement on respiration is done. For toxicity tests animals should be carefully selected in the same size range as will be afterwards used for measurements.

Copper sulfate $CuSO_4$

Table 1: Copper sulfate chemical fact sheet (Merck)

Common name	Copper sulfate
Stehiometric formula	$CuSO_4$
Molecular weight	249.7 g mol^{-1}
Form	Blue or green to white crystals
Odor	No specific odor
Temperature of melting	110 °C
Water solubility	230 g l^{-1} at 20 °C

Trade names are Agritox®, Basicap®, Bluestone®, BSC Copper Fungicide®, CP Basic Sulfate® and Tri-Basic Copper Sulfate®.

First of allochthonous substances selected was copper sulfate ($CuSO_4$) which can be found as a solution in most running waters. Copper sulfate is in large amounts used as fungicide in agriculture, especially in viticulture as an agent to protect grapes against moulds and bacteria. It is used to control diseases of fruit, vegetable, nut and field crops. Diseases that are controlled by this fungicide include mildew, leaf spots, blights and apple scab. It is used in combination with lime and water as a protective fungicide for leaf application and seed treatment. It is also used as an algaecide and herbicide in irrigation and municipal wastewater water treatment systems, and as a molluscicide, a material used to repel and kill slugs and snails. Copper sulfate is a naturally-occurring inorganic salt. However, copper is an essential trace element in plant and animal nutrition (Hartley and Kidd, 1983).

It was selected because of seasonal applications; very high instant concentrations can be locally observed in freshwaters (Boulanger and Nikolaidis, 2003; de Oliveira-Filho et al., 2004). Most important information was that copper can affect mitochondria – site of electron transport system (ETS).

Chronic exposure of animals to copper presents important factor for mortality of specimens of *A. aquaticus and G. fossarum*. Influence of toxic discharges was tested on animals and body metal concentrations were measured. Copper was identified as most important metal toxicant with respect to mortality (Mulliss et al*.,* 1994; Mulliss et al*.,* 1996). Studies of acute toxicity are limited in number and much more data can be found for chronic exposures, so more experiments with heavy metals should be performed after short pulse exposures (Brown, 1976).

1.7.3 Neonicotinoids

Neonicotinoids are the newest class of insecticides that are similar to tobacco toxin nicotine. They are very commonly used because of their selectivity; they are focused mainly on insects. Their structure is different to other synthetic pesticides, including those used on plant origin. In fact they are substitutes for organophosphates and methylcarbamate pesticides basically used for controlling of plant sucking insect pests (Ware and Whitacre, 2004).

Neonicotinoids are named by their site of action that is nicotinic acetylcholine receptors that are more common in central nervous system of insects. They are highly effective on insects that developed resistance to other conventional pesticides. They have very low toxicity to warm-blooded animals and are possibly the most used group of insecticides in the world (Tomizawa and Casida, 2003; Ware and Whitacre, 2004; Guzsvany et al., 2006).

Imidacloprid $C_9H_{10}ClN_5O_2$

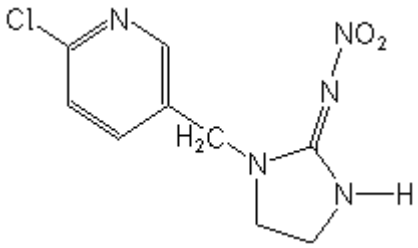

***Figure 6**: Imidacloprid: $C_9H_{10}ClN_5O_2$*

Table 2: Imidacloprid chemical fact sheet (Krohn and Hellpointner, 2002)

Common name	Imidacloprid
IUPAC name	1-[(6-chloro-3-pyridinil)methyl]-N-nitro-2-imidazolidinimine
Stehiometric formula	$C_9H_{10}ClN_5O_2$
Molecular weight	255.7 g mol^{-1}
Form	Colorless crystals
Odor	No specific odor
Temperature of melting	144 °C
Water solubility	610 mg l^{-1} at 20 °C
Henry's constant	$6.5x10^{-11}$atm m^3 mol^{-1} at 20 °C
Adsorption coefficient K_{oc}	225 (from 132 to 310)
Octanol-water coefficient K_{ow}	3.27 at 21°C (log P_{ow} = 0.57)
Vapor pressure	$2x10^{-7}$ Pa at 20 °C
Hydrolysis half-life	>30 days at 20 °C and pH 7
Aqueous photolysis half-life	$3.98x10^{-1}$ days at 24 °C and pH 7
Aerobic soil half-life	from 26.5 to 229 days

The products trade names are Admire®, Confidor®, Gaucho®, Premier®, Premise®, Provado®, and Marathon®; they all contain imidacloprid as the active ingredient (Bacey, 2000; Krohn and Hellpointner, 2002).

Imidacloprid (**Figure 6**) is a broad spectrum systemic chloro-nicotinyl insecticide used as foliar treatment, seed dressing or soil treatment in different crops. The most common use is in rice, cereal, maize, potatoes, vegetables, sugar beets, peanuts, pome fruits, cotton, hops, pecans and turf. It is used for control of a wide range of sucking and biting insects like rice hoppers (*Nilaparvata* sp.: Delphacidae), aphids (Aphididae), thrips (Thripidae: Thysanoptera), whiteflies (Aleyrodoidea: Homoptera), termites (Rhinotermitidae: Isoptera), turf insects (Insecta), soil insects (Insecta) and some beetles (Coleoptera). It can work against insect pests either with ingestion or just by contact. It has also great residual control, which means that imidacloprid as a modern pesticide is active inside plant for longer time after application also at low application concentrations (Moza et al., 1998; Krohn and Hellpointner, 2002).

Mechanism of selective action

Site of action in insects is interference with transmission of stimuli in nervous system. They act as agonists by binding to nicotinic acetylcholine receptors (nAchRs) on the postsynaptic membrane of insects. That results in accumulation of neurotransmitter acetylcholine (Ach) and furthermore in over-stimulation of insect leading in paralysis that can end up as death (Liu and Casida, 1993).

These receptors are much more abundant in insects than in warm blooded animals, consequently they are more selective in action. Imidacloprid has electronegative tip consisting of a nitro pharmacophore that presumably bind to a unique cationic subsite of the insect receptor (Tomizawa and Casida, 2003).

This insecticide is fairly soluble in water compared to other non-polar insecticides (Sarkar et al., 1999). Imidacloprid is not reported as carcinogenuous and presents LC_{50} for mammals at 450 mg kg^{-1} for acute toxicity and for no observed adverse effect level (NOAEL) at 5.7 mg l^{-1} day^{-1}. For birds this dose is 31 mg kg^{-1} and for fish 211 mg l^{-1} (Krohn and Hellpointner, 2002).

There are no specific antidotes for poisoning with imidacloprid (also for all other neonicotinoids) (Tomizawa and Casida, 2003).

Degradation pathways

Imidacloprid has very weak basic properties. Solubility in water and partition coefficient in octanol-water is not influenced by the pH-values between 4 and 9. Imidacloprid can be classified as a hydrophilic substance since water solubility is quite high and a log P_{OW} is low (0.56), it has no potential for bioaccumulation (Bacey, 2000; Krohn and Hellpointner, 2002).

The aerobic degradation of imidacloprid has half-life disappearance time in an average of 156 days. Afterwards imidacloprid should be totally degraded and mineralized to carbon dioxide and the soil matrix intake residues.

Anaerobic conditions and sunlight accelerate the degradation of imidacloprid so in the soil only parent compound can be found and not degradation products. That was proved with numerous field trials in Europe and in the USA, showing no accumulation in the soil. The DT_{50} value reported in Europe are on average 96 days and in the USA between 7 and 146 days (Krohn and Hellpointner, 2002).

The adsorption characteristics of imidacloprid in the soil show high degree of binding to the soil and it is not easily released, so the compound remains in the upper root zone and the translocation to deeper-lying soil zones is negligible (Krohn and Hellpointner, 2002; Guzsvany et al., 2006).

Imidacloprid is not stabile in a surface aqueous environment because of sunlight that accelerates the degradation, the same stands for fog and rain. Low vapor pressure also excludes volatilization from treated surfaces (Moza et al., 1998; Krohn and Hellpointner, 2002).

Hydrolysis of imidacloprid (**Figure 7**) can range from 33 up to 44 days at 25 °C and pH 7 (Zheng and Liu, 1999). Half-life photolysis in water is less than 3 days (Moza et al., 1998). On the soil surface it takes 39 days to the same level, but in the soil it may vary from 27 to 229 days. That persistence is important for longer availability of substance for the root uptake (Bacey, 2000).

Stability of imidacloprid in neutral and acidic conditions is high, due to low hydrolysis. Under basic conditions and high temperature hydrolysis is faster and hydrolysis gives only one main reaction product, 1-[(6-chloro-3-pyridinyl)methyl]-2-imidazolidone (Zheng and Liu, 1999; Sarkar et al., 1999).

Song et al. (1997) with his results suggests that imidacloprid, as a selective insecticide, can be used with a reasonable environmental safety regarding freshwater crustaceans.

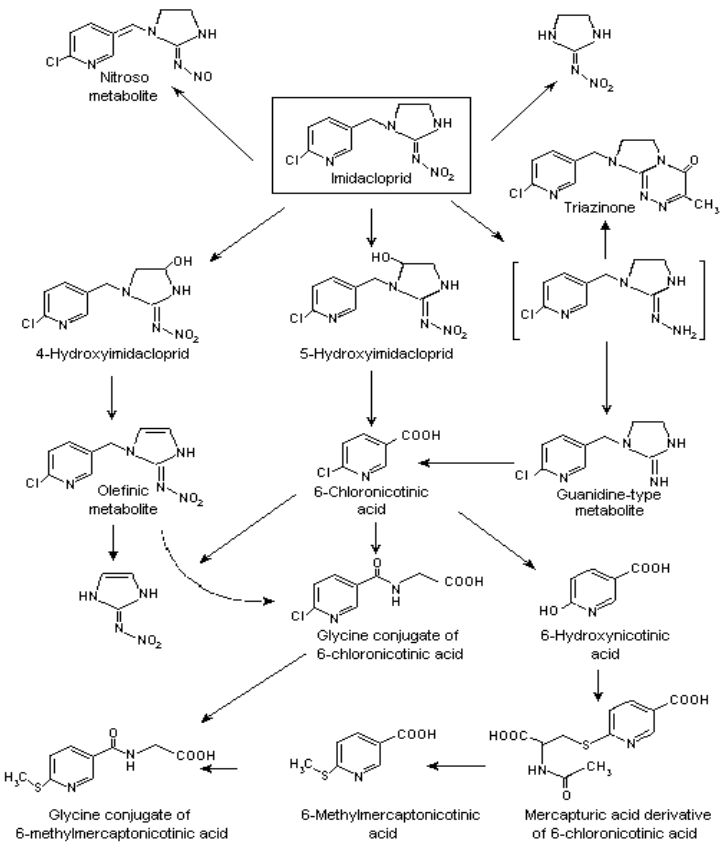

***Figure 7**: Degradation photoproducts of imidacloprid (Moza et al., 1998)*

1.7.4 Triazines

Commercial herbicides like triazines kill weeds by interfering with light reactions of photosynthesis. Inhibitors of photosystem I divert electrons from the terminal part, whereas inhibitors of photosystem II block electron flow of the photosystem. Photosystem II inhibitors include urea derivatives such as diuron and triazine derivatives such as atrazine. They bind specifically to plastoquinone-binding protein D1, one of the two proteins that compose the PSII reaction center. 1,3,5-triazines or *S*-triazines block electron transfer by inhibiting the binding of an oxidized plastoquinone to its binding site on PSII, no electrons can be provided to the electron transport system from PSII (Berg et al., 2002).

For example in atrazine-resistant mutants, a single amino acid change in D1, changes it that much that it is not able to bind the herbicide, so photosynthesis proceeds at normal rates. Such resistant weeds present a major agricultural problem (Berg et al., 2002).

Atrazine: $C_8H_{14}ClN_5$

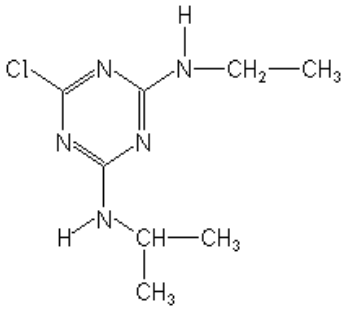

***Figure 8**: Atrazine: $C_8H_{14}ClN_5$*

Table 3: Atrazine chemical fact sheet (Eisler, 1989)

Common name	Atrazine
IUPAC name	2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine
Stehiometric formula	$C_8H_{14}ClN_5$
Molecular weight	215.7 g mol^{-1}
Form	White non-combustible, non-corrosive crystals
Water solubility	32 mg l^{-1} at 25 °C
Henry's constant	$6.13x10^{-8}$atm m^3 mol^{-1} at 20 °C
Vapor pressure	$3x10^{-7}$ Pa at 20 °C
Hydrolysis half-life	>30 days at 20 °C and pH 7
Aqueous photolysis half-life	$3.98x10^{-1}$ days at 24 °C and pH 7
Aerobic soil half-life	from 26.5 to 229 days

Atrazine is only slightly soluble in water but soluble in many organic solvents (360 - 183,000 mg l^{-1}). For methanol solubility is 18,000 mg l^{-1} at 27 °C (Eisler, 1989).

Trade names include Aatrex®, Aktikon®, Alazine®, Atred®, Atranex®, Atrataf®, Atratol®, Azinotox®, Crisazina®, Farmco Atrazine®, G-30027®, Gesaprim®, Giffex 4L®, Malermais®, Primatol®, Simazat®, and Zeapos® (Eisler, 1989).

Atrazine is most commonly used pesticide in North America. In USA alone 50 thousand tons are applied to more than 25 million ha of fields mostly to control broadleaf or grass weeds in corn, sorghum summer fallow and sugarcane (Eisler, 1989). It is still one of the most widely used agricultural pesticides in United States, as 75% of the field corn acreage is treated with atrazine (EPA US GOV, 2003).

Herbicide atrazine (**Figure 8**) acts as inhibitor of photosynthesis (photosystem II) and is relatively mobile and persistent but it does not bioaccumulate in food chains.

Atrazine inhibits photosynthesis, which may result in chlorosis of plant leaves followed by necrosis of leaf tissue. A secondary substance formed as a result of photosynthesis inhibition may be responsible for plant death. When PSII inhibitor is applied to the leaves, uptake occurs into the leaf but very little movement out of the leaf occurs. Injury to corn occurs as yellowing of leaf margins and tips followed by browning, whereas injury to soybean occurs as yellowing or burning of outer leaf margins. The entire leaf may turn yellow, but veins usually remain somewhat green (interveinal chlorosis). Lower leaves are most affected, and new leaves may be unaffected. Atrazine generally is absorbed both by roots and foliage (Eisler, 1989).

Atrazine breaks down to other compounds by abiotic (chemical breakdown) and also biotic (microbial transformation) factors. Those secondary compounds can be also toxic to environment though usually less than original pesticide compounds (Solomon et al., 1996).

Concentrations of this chemical in agricultural watersheds in small streams and rivers are usually episodic and peaks are in accordance with main pesticide application. Usual pre-season handling and application with atrazine is in May and June (Solomon et al., 1996).

Degradation pathways

Atrazine is stable for several years in manufacturer's products containing around 80% of pure substance produced as powders, pellets, granules, flowable concentrates or tablets. Degradation of atrazine begins immediately after application (Eisler, 1989).

Three main degradation pathways are named after site of molecule breakdown that can be on different carbon atom. Three ways are hydrolysis of atom 2, 4, or 6 of the atrazine ring.
For hydrolysis at carbon atom 2; chlorine atom is replaced with a hydroxyl group and product is named afterwards as **hydroxyatrazine** (2-hydroxy-4-(ethylamino)-6-(isopropilamino)-s-triazine). Loss of ethylpropyl group at carbon atom 4; the product is **desethylatrazine** (2-chloro-4-amino-6-(isopropylamino) -s-triazine). Loss of isoprophly group at carbon atom 6; as **desisopropylatrazine** (2chloro-4-(ethylamino)-6-amino-s-triazine). Temperature is also important factor, since atrazine concentration decreases 3 to 4 times faster in soils at 25 °C than at 10 °C (Qiao et al., 1996).

Microbial transformation of atrazine usually brings desethylatrazine and desisopropylatrazine, while chemical degradation yields as hydroxyatrazine (Eisler, 1989; Qiao et al., 1996). Those degradation products are less toxic than atrazine. In fact Huckins et al. (1986) reported that in less than four days after application atrazine rapidly disappeared from freshwater microcosm in Northern Prairie.

In beginning atrazine was reported environmentally safe regarding the case of lateral and downward movement of atrazine. Since atrazine adsorbs onto certain soil constituents depending on the moisture and type of the soil that may vary its availability also for next year. Adverse effects were detected only right after application, but long term population effects on fauna was considered negligible. Atrazine was said not to be detected below the upper 30 cm of soil even after years of continuous use. That is why atrazine was not expected to be found in freshwaters at recommended application rates. Sensitive aquatic species might still experience temporary adverse effects at quite low concentrations on flora up to 20 µg l^{-1} and fauna from 94 to 500 µg l^{-1} (Eisler, 1989).

Ritter et al. (1994) confirmed that atrazine can be detected in groundwater since it moves rapidly downward into the soil if sufficient rainfall occurs shortly after application. Atrazine concentrations can range from 1 to 54 µg l^{-1} in groundwater under corn field that is continuously irrigated. Measured values are outside the Federal Maximum Contaminant Level (MCL) for drinking water that is 3 µg l^{-1}. Peak of atrazine was reached after 59 days after application and it was gone under detectable value only after 200 days.

Ferrari et al. (1997) reported that pesticides can be found also in surface waters in Mid-Atlantic region in USA. They detected pesticides in more than 90 percent of sampling sites tested. Greater atrazine concentrations than the Federal MCL for drinking water were found in 67 of 2,076 samples analyzed.

Unexpected vertical and horizontal transport of atrazine and its persistence, as well as its adverse effects al low concentration led to ban of the sell in several countries, also in Slovenia (OJ of RS 29/1996).

In United States there is a new reassessment and re-registration of atrazine as a latest step in a process to review atrazine and other older pesticides against current standards. They introduced intensive monitoring program for raw water with weekly sampling during the pesticide use season and biweekly for the rest of the year. Risk assessment from year 2002 emphasized that atrazine exposure below the standard level is not carcinogenic to human (EPA US GOV, 2003).

1.7.5 Pyrethroids

Pyrethrum from natural source has rarely been used in agriculture as pest control because of its instability in sunlight and price. Synthetic pyrethroids are generally more effective against insect pests in low application rates and are much more stable in sunlight. In last decade there was an evolution of a wide range of synthetic pyrethroids. Four generations of insecticides were produced till present.

The **first** generation is only one pyrethroid – allethrin (Pynamin®) that was introduced back in year 1949. Its synthesis was very complicated involving 22 chemical reactions (Laskowski, 2002).

The **second** generation consists of tetramethrin (Neo-Pynamin®) from year 1965, resmethrin (Syntherin®) and bioresmethrin from 1967, Bioalletrhin® from 1969 and phonothrin (Sumithrin®) from year 1976. Bioresmethrin is 50- while resmethrin is 20-times as effective as pyrethrum (E.I. duPont de Neumors, 2002).

In the **third** generation there are fenvalerate (Pydrin®, Tribute®, Bellmark®) and permethrin (Astro®, Ambush®, Dragnet®, Flee®, Pounce®, Prelude®, Talcord® and Torpedo®) they all appeared in 1972 to 1973. They became number one of pyrethroids because of their exceptional activity and photostability (E.I. duPont de Neumors, 2002).

The **fourth** and current generation, is even more effective. These include bifenthrin (Capture®, Talstar®), *lambda*-cyhalothrin (Demand®, Karate®, Scimitar® and Warrior®), cypermethrin (Ammo®, Barricade®, Cymbush®, Cynoff® and Ripcord®), cyfluthrin (Baythroid®, Countdown®, Cylense®, Laser® & Tempo®), deltamethrin (Decis®) **esfenvalerate** (**Figure 9**) that was used in our experiments (Asana®, Hallmark®, Sumi-Alfa®), fenpropathrin (Danitol®), flucythrinate (Cybolt®, Payoff®), fluvalinate (Mavrik®, Spur®), prallethrin (Etoc®), *tau*-fluvalinate (Mavrik®) tefluthrin (Evict®, Fireban®, Force® and Raze®), tralomethrin (Scout X-TRA®, Tralex®), and *zeta*-cypermethrin (Mustang® and Fury®). Recent additions to the **fourth** generation pyrethroids are acrinathrin (Rufast®), imiprothrin (Pralle®), registered in 1998, and *gamma*-cyhalothrim (Pytech®), which is in development. They are all synthesized to be effective, photostable with minimal volatility which enables them up to 10 days residual efficiency (E.I. duPont de Neumors, 2002).

The pyrethroids have similar action site as DDT and are axonic poisons. Their action is modifying of the gateing characteristics of voltage-sensitive sodium channels in mammalian and invertebrate neuronal membranes with delaying of their closure. They are dissolved in the lipid phase of the membrane and bind to a receptor site on the alpha sub-unit of the sodium channel. There are two types of pyrethroids regarding changes in ambient temperature. Type I with a negative temperature coefficient (similar to DDT). Type II has a positive temperature coefficient, showing increased kill with increase in ambient temperature (Laskowski, 2002).

Pyrethroids act on both the peripheral and central nervous system of the insect pest. They cause paralysis with stimulation of nerve cells with production of repetitive discharges. Excitation is caused by sodium ions that enter the axon through tiny hole in sodium channel made by pyrethroids. This stimulation is greater than that of pesticide DDT (Ware and Whitacre, 2004).

Pyrethroid insecticides are primarily used for controlling of agricultural pests in various crops. They are recognized to be highly toxic to aquatic invertebrates (Lozano et al., 1992).

Esfenvalerate $C_{25}H_{22}ClNO_3$

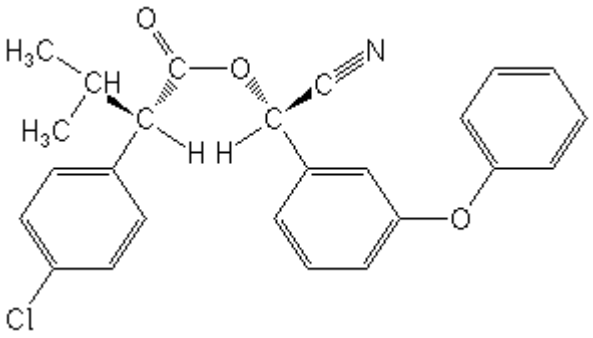

***Figure 9**: Esfenvalerate: $C_{25}H_{22}ClNO_3$*

Table 4: Esfenvalerate chemical fact sheet (Wauchope et al., 1992)

Common name	Esfenvalerate
IUPAC name	(S)-α-cyano-3-phenoxybenzil(S)-2-(4-chlorophenyl)-3-methylbutyrate
Stehiometric formula	$C_{25}H_{22}ClNO_3$
Molecular weight	419.9 g mol^{-1}
Form	Colorless crystals
Water solubility	Less than 0.002 mg l^{-1} at 25 °C
Henry's constant	$1.4x10^{-12}$atm m^3 mol^{-1} at 25 °C
Vapor pressure	0.067 mPa at 25 °C
Hydrolysis half-life	65 to 129 days at 20 °C (from pH=5 to 9)
Aqueous photolysis half-life	21 days at 20 °C
Aerobic soil half-life	72.3 (15 to 90) days at 10 °C

Trade names for esfenvalerate are Asana®XL, Halmark® and Sumi-Alfa® (Wauchope et al., 1992).

Esfenvalerate is a synthetic insecticide that is used against moths, flies, beetles and other insects. It is used on vegetable like sugarbeets and tomatoes, further to nut crops and tree fruits (Wauchope et al., 1992). For wide range use it is usually mixed with a variety of other pesticides like carbamates or organophosphates.

Esfenvalerate is more toxic since it requires lower application rates and it is very similar compound to fenvalerate. Both are active ingredients of insecticides (Fairchild et al., 1992). The only difference is higher relative proportion of insecticidally active S,S - isomer that makes esfenvalerate more powerful insecticide and also less chronically toxic (Wauchope et al., 1992; Laskowski, 2002; Graebing and Chib, 2004).

Esfenvalerate is by application in several countries one of the highest ranked among all pesticides used in agriculture. It is declared as highly toxic to freshwater animals like fish (concentrations from 0.3 to 1 µg l^{-1}) and *Daphnia magna* (1 µg l^{-1}). In Danish streams concentrations up to 0.66 µg l^{-1} were reported (Aarhus Amt. 1999, cit. by Forbes and Cold, 2005).

Short term exposures to esfenvalerate alone showed effect on animals also after 2 weeks (Cold and Forbes, 2004).

Degradation pathways

The stability of esfenvalerate in sunlight allows its application against a wide range of pests. Residue levels are minimized by low application rates and poor translocation characteristics in plants and in soil. In freshwater esfenvalerate is fairly quickly degraded as its half-life is 21 days due to sunlight effect. In soil it is moderately persistent with a half-life from 15 to 90 days depending on the soil type. Esfenvalerate and its breakdown products are relatively immobile in soil, which reduces a risk for accumulation in groundwater and in sediment (Wauchope et al., 1992; Graebing and Chib, 2004).

The European Commission, Directorate-General Health & Consumer Protection (Pan-Europe, 2000), reported the photostability (DT50) of esfenvalerate in water is 10 days when exposed to sunlight and 6 days when exposed to artificial sunlight. Hydrolytic stability of esfenvalerate was reported to be 129 days at pH 5 and 65 days at pH 9. Limited hydrolysis was reported at pH 7. Laskowski (2002) reported that esfenvalerate was stable to hydrolysis at three pH values (5, 7 and 9). A relatively slow rate of degradation of esfenvalerate in aerobic aquatic systems and determined a half-life for aerobic degradation to average 72.3 days (range 65-79 days) in water incubated without light at 10 °C for 100 days. 90% of esfenvalerate was lost from the water column (adsorbed to particulates) within 24 hours following application to littoral enclosures. Volatilization is not expected to play a role in esfenvalerate disappearance from water due to its very low Henry's law constant (Laskowski, 2002).

No data on acute or chronic toxicity of this pesticide for our selected test organisms could be found in references. Since *G. fossarum* and *A. aquaticus* are nontarget species, no work has been accomplished in this field before.

1.8 Stress enzymes

Metabolism of most of allochthonous substances runs in two steps. First part is oxidation, hydrolysis, hydration or reduction to primary metabolite which has hydroxyl group. In the second phase biotransformation takes place, those metabolites bond with different water soluble endogenuous substances (oligo-saharids, amino-acids and peptides). Most of those biotransformations mean detoxification processes for living cells (Storey, 1996; Walker et al., 1996).

Oxidative stress

Aerobic metabolism in cells generates potentially dangerous reactive oxygen metabolites that are formed by oxygen (O_2) reduction. Normal byproducts in one electron reduction of oxygen produce free radical superoxide ($\cdot O_2^-$) whereas two electron reductions produce highly reactive oxygen species hydrogen peroxide (H_2O_2). Three electron reductions formed by Fenton reaction is hydroxyl radical ($\cdot OH$) which is also extremely reactive. These electrophilic compounds are harmful to living cells. Usually functional processes in cells are tightly coupled, in order to prevent many reductions and thus reduce contact-time of reduction processes with cellular structural and functional elements. The reactive electrophilic groups can harm cells content like proteins, DNA and lipids; also those in membranes. Free radicals like hydroxyl radicals ($\cdot OH$) can cause protein peroxidation, DNA damage and lipid hydroperoxidation (Ahmad et al., 1989). Several enzymes are able to cope with harmful effects of reactive electrophilic groups (Storey, 1996; Walker et al., 1996; Cairrão et al., 2004).

Superoxide dismutase (SOD) is enzyme that copes with superoxide ($\cdot O_2^-$) and transforms it to free hydrogen peroxide. There are two detoxification pathways with two enzymes dealing further with it – reducing it to water. First is catalase and the second one glutathione peroxidase (which needs co-enzyme and another ½ O_2) (Ahmad et al., 1989; Fenton and Summers, 1995).

Glutathione conjugation is considered to be one of protective compounds that may be naturally occurring or come from metabolic pathways (Lamoureux and Rusness, 1989; Voet and Voet, 2006).

Glutathione (**Figure 10**) is antioxidant tripeptide compound soluble in water. It acts as a nonspecific reducing agent, keeping proteins in their reduced thiol form – preventing cysteine residues from oxidizing and forming disulphide bridges (Walker et al., 1996).

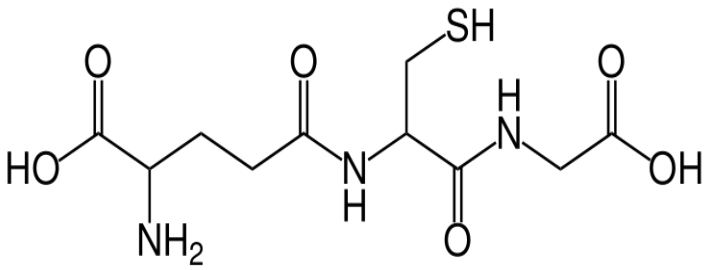

***Figure 10**: Glutathione: $C_{10}H_{17}N_3O_6S$*

Glutathione is involved in two types of conjugation reactions. (a) Displacement reactions; where glutathione displaces an electron-withdrawing group, such as halogens, nitriles and carboxylic acids.
(b) Addition reactions; where glutathione is added to activated double bond structures or strained ring systems i.e. nucleophilic addition (Habig et al., 1974).

Glutathione peroxidase (GPOx)

This group of enzymes has a main biological role in protection of the organism from oxidative damage. Biochemical function is to reduce free hydroperoxide to water and lipid hydroperoxides to alcohols. This tetrameric glycoprotein (four selenocysteine amino acid residues) is selenium dependant (Vontas et al., 2001).

Main reaction is:

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

Glutathione reductase is the enzyme that provides new glutathione molecule from glutathione disulphide (GSSG) with energy from NADPH.

Second reaction that protects cellular and subcellular membranes catalyzes lipid hydroperoxides reduction (Prohaska, 1980).

Glutathione S-transferase (GST)

Transferases are defined as enzymes that catalyze the transfer of one functional group from one molecule (donor) to another (acceptor) (Voet and Voet, 2006). GSTs may also bind toxins and function as transport proteins and because of that an early name was “ligandin”.

GSTs are a complex group of enzymes which mediate the conjugation of compounds with glutathione. This activity is useful in the detoxification of endogenous compounds such as peroxidized lipids, as well as for the metabolism of xenobiotics. GST has catalytic power for producing thioether bond between cysteine on the glutathione with electrophilic compound. Product of this reaction is less reactive and more soluble in water. Potential compounds that are substrates for GST are pesticides and their metabolites, other environmental pollutants and carcinogenic products.Whole process consists of two phases in which first phase is conjugation. This enzyme system is not able to finish their metabolism, but it helps with conversions to cysteine and mercapturic acid conjugates that are more excretable substances. Other electrophilic xenobiotics like epoxides, aldehydes, quinones, hydroperoxides, etc, are also in a chain of potential substrates for the GST group (Habig et al., 1974; Stenersen et al., 1987; Falkner and Clark, 1992).

2. MATERIAL AND METHODS

2.1 Materials

2.1.1 Origin of test animals for culture stock

Animals were collected by the kick sample method according to Hynes (1954). Hand net with mesh size of 0.5 mm was used for collecting animals from a water body (pool or river) (**Figure 11**). Samples with animals were put in plastic buckets filled with water from collecting site (ratio between sample volume and water was 1:4). During the one hour transport to the laboratory buckets were held in cool box. In the laboratory mixed water with sample was put into plastic trays and plastic spoons were used to pick up individual animal. For all further experiments animals were held in monocultures in plastic trays filled with combination of water from collecting site and synthetically prepared water.

***Figure 11**: Collecting of animals from spring Mrzlk at Zadnji kraj (Lake Cerkniško jezero)*

Specimen of water lice (*Asellus aquaticus* L.) were collected from location Zadnji kraj, which is part of the intermittent Lake Cerkniško jezero, where there is low or no rural activity. Animals were collected from the banks of the spring Mrzlk which is active through all of the year and it is just above the mean level of the lake itself. Watershed of the spring is not well defined due karst geology, which is covered with mixed deciduous/coniferous forest. The intermittent lake that is disappearing and appearing again influences also the spring, but due to its karstic origin, spring is active throughout the year. The nature of the collecting site changed every time we collected animals (summer 2006; see the **Figure 11**). In spring time water level was usually quite high, while in summer and autumn only a slow current in very shallow water was found with enormous quantities of animals.

Specimens of side-swimmer (*Gammarus fossarum* (Koch)) for the first experiments were collected also by the kick sample method from the Iščica River. Sampling site is located on the field north to the village Ig and close to the bridge, about 1 km from the settlement. Last two years (2006 and 2007) the alternative animal collection site was near village Duplje near Naklo (Kranj). Small waterbed just outside of the cave Dupeljska jama is good location for *G. fossarum*, since animals are numerous through out whole year. The water flow (cca. 0.1 m s^{-1}) and temperature (around 8 – 9 °C) are almost constant as the water is slowly coming from the cave. For transport to the laboratory the animals were transported inside cool box in plastic tanks (35 x 25 x 5 cm) filled with water from the collection site.

Food

Leaves of black alder (*Alnus glutinosa* (L.) Gaertn.) were used for food. They were collected in late autumn just before fall from black alder trees around Biotechnical Faculty in Ljubljana. In the laboratory they were washed with distilled water and dried between clean filter papers. Dried leaves were stored in the laboratory at the room temperature. At least one week before they were offered to animals they were preconditioned in synthetic water. In order to give a source of colonization of a new set of dried leaves, few leaves already colonized with fungi and bacteria were added to the soaked new ones. Preferred food for test animals are not alder leaves alone, but the biofilm that grows on the surface of those leaves.

2.1.2 Chemicals used for exposure solutions and culturing

Copper sulfate

Copper sulfate anhydrous ($CuSO_4$) was obtained from Merck. Solutions with different concentrations were prepared in double distilled water. They were diluted from stock solution with 1000 mg l^{-1} Cu^{2+}. Stock solution was prepared and stored in glass medicine bottle at 4 °C. To 0.5 l of the stock solution one drop of analar nitric acid was added, to lower pH value and to reduce precipitation/absorption of the metal ions (Martin and Holdich, 1986).

Exposure time of 10 individuals of *A. aquaticus* per each concentration was 96 h. For WRFTT nominal test concentrations were 0.1, 1, 10, 100, 1000 mg l^{-1} Cu^{2+}. For the DATT nominal concentrations used were 5, 10, 20, 30, 40 mg l^{-1} Cu^{2+}. LC_{50} was determined and according to it, experimental exposure concentrations selected were 3, 5 and 10 mg l^{-1} Cu^{2+}. Exposure time for those concentrations was 24 hours, after that oxygen consumption measurements were done in Cyclobios microrespirometer.

Imidacloprid

Original pesticide Confidor SL 200 was used and stock solution was prepared in bi-distilled water containing 200 g l^{-1} of imidacloprid. Original bottle with aqueous soluble concentrate was obtained from Pinus d.d. (Bayer CS d.o.o.) and stored at 4 °C.
Test animals were *A. aquaticus* and *G. fossarum*.
For WRFTT nominal test concentrations were 0.01, 0.1, 1, 10, 100 mg l^{-1} of imidacloprid. DATT nominal concentrations used were 1, 3, 10, 30, 100 mg l^{-1} of imidacloprid.

Atrazine

Analytical standard 99.9% (M = 215.69 g mol^{-1}) was used. Acquired from Riedel-de Haën; as 35702 Pestnal® (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine). Analytical standard, and not any specific trade mark, was used in our experiments. Stock solution was prepared and calculated in methanol with calculated initial concentration for the first exposure solution that should not exceed 1% of methanol. Prepared stock solution contained 100 mg l^{-1} of atrazine.

For WRFTT nominal test concentrations used were 0.01, 0.1, 1, 10, 100 mg l^{-1} of atrazine. The highest concentration used for WRFTT was 100 mg l^{-1} that contained also 10% of methanol. A negative control solution containing only 10% of methanol was used to check for mortality caused by solvent faze. For the DATT nominal concentrations used were 0.3, 1, 3, 10, 30 mg l^{-1} of atrazine.

Selected exposure concentrations for *A. aquaticus* were 5 and 10 mg l^{-1}, while for *G. fossarum* 1, 3 and 10 mg l^{-1} of atrazine. All oxygen consumption measurements were done in new respirometer with OXY-4.

Esfenvalerate

Powder form of (S)-α-cyano-3-phenoxybenzil(S)-2-(4-chlorophenyl)-3-methylbutyrate was obtained from Sigma-Aldrich (Vallensbæk Strand, DK) with technical grade 99.9%. Stock solution (c = 0.1mg l^{-1}) was prepared in acetone and all used concentrations were diluted from this stock solution in synthetic water.

Selected test animal was *G. fossarum*. Several experiments with stress enzymes were conducted in the laboratory in Roskilde (Denmark) in previous years with local species *G. pulex*; we tried to compare reactions of exposure to esfenvalerate on those two species. Since we developed a new method with microcalorimeter, which was not done before, we were able to conduct measurements only with *G. fossarum* and not also with *A. aquaticus.*

Stock solution was prepared as 1 mg of esfenvalerate diluted in 1 ml of acetone (technical grade 99.8%). Start esfenvalerate concentration was 1 g l^{-1} and all consequent solutions were prepared from the stock solution. Final acetone concentration in test solution never exceeded 1:1000 v/v. Solvent effect was tested in separate control group using only the highest amount of the acetone used.

Due to limited number of animals brought from Slovenia WRFTT was not performed. Quick DATT was done instead with test concentrations proposed from former experiments from the laboratory in Roskilde for *G. pulex* (pers. com. Nielsen). Exposure concentrations used were 0.02, 0.1, 0.2, 0.3 µg l^{-1} and control containing only synthetically prepared water.

Synthetic water preparation

Chemical composition of the water for all experiments was obtained from double distilled water and minerals. Proper pH was also obtained with adding prescribed amounts of minerals (ISO-standard 6341, 1996). Water for animal laboratory culture and for all experiments was prepared prior use and pre-cooled to selected temperature for direct use.

Table 5: Synthetic water preparation protocol (ISO-standard 6341, 1996)

For every 10 l of bi-distilled water was added:	
Calcium chloride-dihydrate $CaCl_2$ x 2 H_2O (Merck)	2940 mg
Magnesium sulfate heptahydrate $MgSO_4$ x $7H_2O$ (Kemika)	1230 mg
Sodium hydrogen carbonate $NaHCO_3$ (Kemika)	650 mg
Potassium chloride KCl (Kemika)	60 mg

Water was than cooled to 10 °C and kept in clean plastic containers prior to use.

2.1.3 Instruments

Cyclobios microrespirometer

Oxygen consumption was estimated in a microrespirometer Twin-Flow (CYCLOBIOS, Innsbruck) (**Figure 12**). Two tanks are filled with synthetic water and 100% saturation is obtained by aeration of both tanks via air pumps (flow of air approx. 30 l h^{-1} for each aeration tank). Average water flow with c. 5 ml h^{-1} is provided by peristaltic pump. Saturated water with oxygen slowly passes through the chamber with an animal. The chamber is 5 mm in diameter and 25 mm long glass tube that ends in two bungs into tubes with smaller diameter. The oxygen concentration is measured with membrane covered polarographic oxygen sensors (POS) (Gnaiger and Forstner, 1983) before and after water enters the tubes. This is electrochemical device in which the steady-state current at working electrode (the cathode) is linearly proportional to the concentration of oxygen in contact with the external surface of the membrane. POS consists of pure platinum wire-sealed ring cathode and silver anode inside. Electrolyte solution (KCl) is fitted between both electrodes and covered with 25 µm teflon-silicone double membrane that comes into contact with outside water. Electrolyte provides medium for oxygen molecules between both electrodes. Computer acquires data directly from microrespirometer and continuously plots them on graph on the screen.

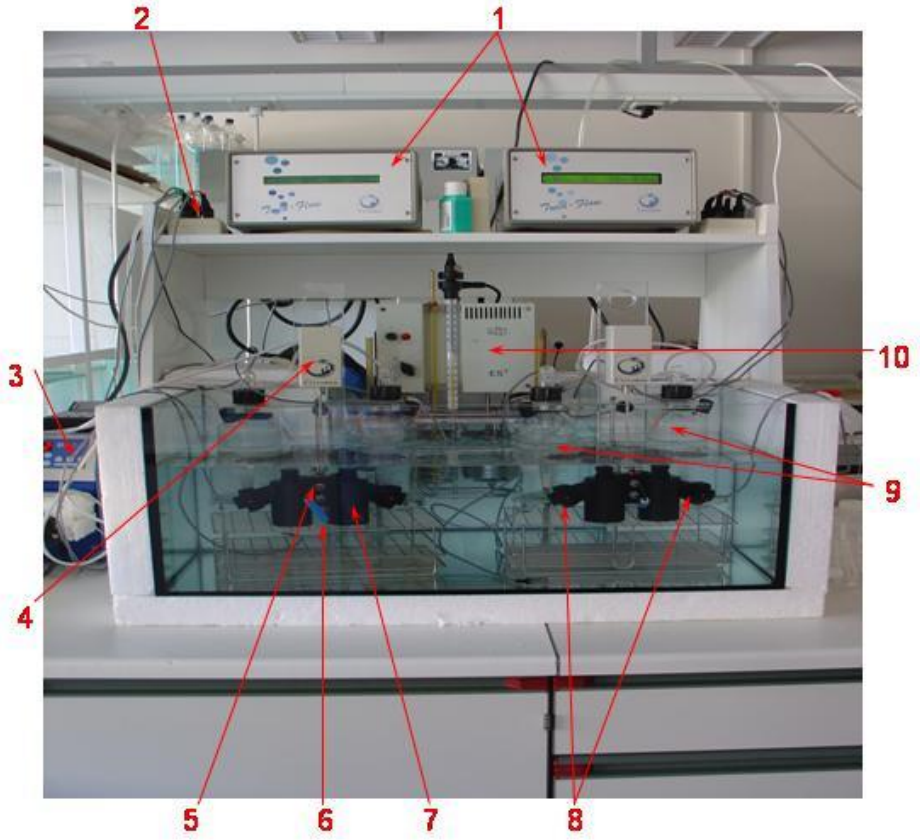

***Figure 12**: Two microrespirometers Twin-Flow (Cyclobios, Innsbruck) in tempered water pool. 1- processing unit of Cyclobios microrespirometer unit with digital display, 2 – plate with connectors for electrodes and for temperature sensor, 3 – peristaltic pump, 4 – automatically or manually handled switch for valve, 5 – body of microrespirometer with inside valve and two cylinders with stirring chambers, 6 – animal test chamber covered with rubber cover, 7 – cylinder with magnet stirrer, 8 – pair of polarographic electrodes inserted to both cylinders 9 – aerating tank with bubble trap, 10 – tempering device for keeping constant temperature in the range of ± 0.05 °C.*

Improvement of oxygen consumption measuring equipment was PreSens OXY-4 introduction (in the **Figure 13**). This is four channel oxygen meter with direct online oxygen measurement. OXY-4 is connected with four 2 mm polymer optical fibers to flow-through fiber-optic oxygen minisensors (all PreSens GmbH). Computer collects data directly from OXY-4 and plots four separate graphs online directly on the screen and data are separately stored in a simple txt files.

The base for this apparatus is water tank from Twin-Flow microrespirometer that provides maximum oxygenated water. Water tank is with vitton tubes connected to first flow through sensor that calibrates the whole system. From the first sensor tube connection splits in three parallel tubes, each into small individual animal chamber (5 mm in diameter and 25 mm long glass tube) each followed by flow-through sensor. Peristaltic pump on the end of the system creates negative pressure which force current of water out of all three tubes (with approx. evacuation of 5 ml h^{-1}). In this improved construction type the system allows independent measurement for tree individual test animals. Calibration is done simultaneously for all three of them with the first sensor, positioned before chambers with animals, thus measuring oxygen concentration of water entering animal chamber. Other three sensors record oxygen concentrations in water leaving the animal chamber.

Data stored in the txt file can be imported into Windows Excel and plotted to a graph. From the graph measured average values of oxygen concentrations are subtracted from average calibration value, obtained from the first sensor. Result is than divided by flow rate measured with volume of water per hour.

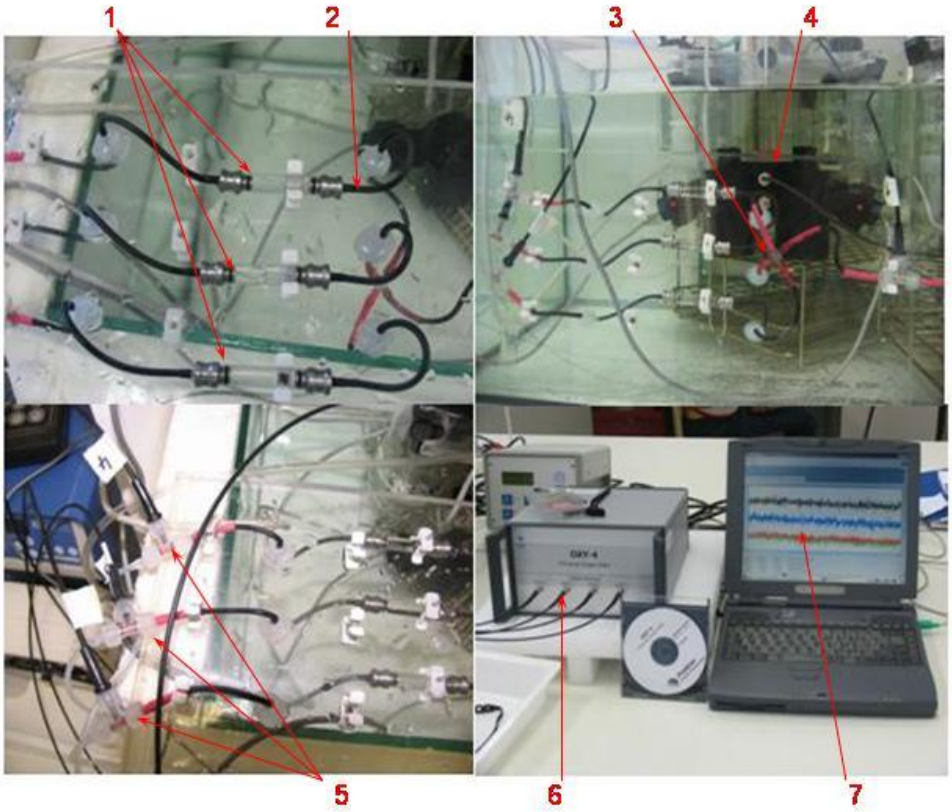

***Figure 13**: Equipment with flow through sensors and OXY – 4 (PreSens GmbH). 1 – test chamber, 2 – vitton tube, 3 – one to three-way crossing, 4 – Cyclobios microrespirometer with aerating tank, 5 – flow-through sensor (FTC-PSt3), 6 – OXY-4 with four connectors leading to 2 mm polymer optical fiber (POF), 7 – 4 graph online plot by PreSens software*

***Figure 14**: Microcalorimeter MC-DSC (Calorimetry Science Corp., Lindon, UT). 1 – one of four isolated calorimeter chambers , 2 – two lids for cover chamber, 3 – reference chamber, 4 – holder for containers and first lids, 5 – steel container, 6 – first threaded lid with a gasket, 7 – tempering pool with antifreeze solution, 8 – last cover lid for all four chambers, 9 – calorimeter*

Microcalorimeter MC-DSC

Microcalorimeter MC-DSC (Calorimetry Science Corp., Lindon, UT) (**Figure 14**); with four TED (Thermal Electric Devices) detectors was used to measure heat rate directly under constant temperature – isothermal mode. Stabile water bath system was used for cooling at least 10 °C below the lowest measuring temperature in each particular experiment.

The calorimeter has 4 removable steel containers with volume of 1 cm^3 (**Figure 14**). First of them was used as thermal reference and other three were used for heat rate measurement with animals. The containers were closed with a threaded lid with a gasket. Containers were handled with clean and dry gloves. Lids on the calorimeter were handled with synthetically coated tweezers. Measurement for every container was simultaneously recorded every 30 seconds.

Room temperature and dry nitrogen were used to prevent condensation outside the containers that could interfere with microcalorimetric measurements (i.e. energy of condensation) at measuring below ambient temperature.

2.2 Experimental set-up

2.2.1 Maintaining test animals in the laboratory

In the laboratory animals of both species were not separated by sex, thus males and females were put together, to breed. Laboratory culture of *A. aquaticus* was established in aquarium tanks (44 x 41 x 7 cm) with separate tank (35 x 17 x 20 cm) for filtering and aerating water. Water was prepared as "synthetical" water, where mineral salts were added to double distilled water to mimic standard natural water. "Synthetic" water (for details see 2.8.1) was prepared regularly with 14 days intervals just before replacement of used water from culture in order to prevent instant algal and fungal spreading.

For experiments only males of both species were collected from culture and before use in the experiment they were put in the tanks with synthetic water at 10 °C and day/night period 12/12h.

At the start of the measurements of oxygen consumption in microrespirometer, filtered water from a natural source was used. It was filtered through membrane filter with pore size of 0.2 µm in order to stop algal growth in the microrespirometer. Later on synthetically prepared water from double distilled water with added minerals was used for culturing, which was also used in all measuring experiments afterwards. ETS and protein content was measured later on when at least six animals were taken out of the microrespirometer.

Later on for the second part of experiments, water ratio 50 : 50 (filtered natural water : synthetic water) for culturing of test animals was used. Whole system was simplified and the test culture were put into plastic trays with approx. three cm of water in a chamber with controlled conditions (temperature and light) and they were kept there for at least ten days for adaptation to temperature used during measurements.

In all experiments test animal chamber was darkened with a rubber cover that fitted glass wall from outside. In microcalorimeter measurement were also done in the dark. All water solutions used in experiments were also kept at the same temperature that was selected for measurements, for reduction of bubbles formation.

2.2.2 Experiments at different temperatures

Measurements at the start were conducted at two different temperatures; 10 and 15 °C. At least 10 days before each experiment, animals were slowly acclimated to selected temperature. The animals were randomly picked out, transferred to clean "synthetic" water and held in a culture until measurements.

For both test species we took population that we used for further experiments as a control group. For *A. aquaticus* control group was from the Lake Cerkniško jezero and for *G. fossarum* specimens from the Iščica River made control group, all measured at 10 °C. For the first test we took animals from the same location and tested them at 15 °C. After that, for the second test, we compared another population from different location at the same temperature (10 °C).

2.2.3 Testing toxic substances

At the start of effective and lethal concentration determination, standard acute toxicity test experiments were performed. Stock solution was prepared from concentrated original substance to selected concentration. Glass medicine bottle was first cleaned and pre-exposed with stock concentration for an hour in order to minimize loss of pesticide due to adsorption to surface (Lee et al., 2002; Forbes and Cold, 2005).

Prepared stock solution at selected concentration was then used for all the experiments for each selected pesticide.

Animals were randomly selected from culture and held without food 24 h before the test. They were put into test vessel (9 x 7 x 5 cm) filled with selected concentration of tested substance. At least one hour before the start of experiment with animals, solution was exchanged in the chambers. All mortality experiments were conducted in controlled conditions in a big test chamber at 10 ± 0.1 °C and a photoperiod of 12 h of night and 12 h of day. Glass vessels were shaded from the side of light source.

Acute toxicity tests were done for four allochthonous substances. Two series for each substance were done, and for each series at least 80 animals were used. Tested animals were compared against control animals that were held under the same test conditions.

For the first wide range finding toxicity test (WRFTT), stock solutions were established to give five concentrations in logarithmic ratio 0.01, 0.1, 1, 10, and 100% of the selected concentration and two controls. One control contained only synthetically prepared water and second water from the culture medium.

The second test was the definitive acute toxicity test (DATT) also with 5 concentrations and both controls. Concentration series were prepared with factor 0.3 (100, 30, 10, 3, 1%) in the range of LC_{50} from the first test. Exposure period was 96 h. Results of test were recorded every 12 h. Test solutions were replaced every 48 h to maintain constant concentrations (De Nicola Giudici et al., 1988). Dead animals were recorded and removed during each exposure. Animals were considered dead if no appendage movement was visible during 15 second observation period.

Effective (EC_{50}) and lethal concentrations (LC_{50}) were defined, as well as no-observed-effect concentration (NOEC). From that point on lowest-observed-effect concentration (LOEC) was used as a measure of concentration for determination of test solutions. Around 10 % up to 100 % of LC_{50} was used as sublethal test solution (Clesceri et al., 1998).

Also before the start of toxicity tests animals were transferred to clean water without food in order to reduce excretion in test chambers that could influence oxygen consumption. Food was not given to reduce differences within animal behavior and reduce differences of oxygen consumption due to digestion.

After all LC_{50} were acquired, different sublethal concentrations were selected and prepared in fresh test vessels. Animals were randomly selected and exposed at start experiment to copper sulfate for 24 h and later on to imidacloprid, atrazine and esfenvalerate for 1 hour – as short term exposure period.

Oxygen consumption was measured in microrespirometer (both types) for each individual animal. Measurements at different temperatures and after exposure to copper sulfate were done in Cyclobios microrespirometer, while most for imidacloprid and all for atrazine were performed on a new respirometer with OXY-4 oxygen meter.

ETS activity and protein content was measured every time when at least six animals were taken out of the microrespirometer. Total protein content measurement method was introduced at experiments with atrazine and esfenvalerate and was not performed in former experiments with copper sulfate or imidacloprid.

Animals were exposed in a plastic Petri dish (Φ = 10cm) containing 33 ml of selected solution (100 ml of solution was mixed every time). Solution was exchanged just before animals were put in for a one hour short pulse exposure. Vessels were covered with a paper to reduce direct light stress for animals that were just taken out of microcalorimeter. After exposure animals were put back to microcalorimeter for another measurement (as explained in 2.3.5).

Cold and Forbes (2004) suggested better explanation of exposure time, they proposed short term one hour exposures. LC_{50} was roughly determined with WRFTT. Selected sublethal exposure concentrations for *A. aquaticus* were 0.01, 0.1, 1 and 10 mg l^{-1} of imidacloprid, while for *G. fossarum* they were 0.01, 0.1 and 10 mg l^{-1} of imidacloprid and control containing only synthetically prepared water. Oxygen consumption measurements for *G. fossarum* were done in Cyclobios microrespirometer, while for *A. aquaticus* they were tested in new respirometer with OXY-4.

2.3 Methods

2.3.1 Wet mass (WW) and dry mass (DW) determination

Wet mass usually in literature cited as wet weight (from now on WW) of each individual test animal was determined on a balance (Sartorius Research) to the nearest 0.1 mg. Before that, animal was gently pressed against the paper towel in order to reduce water from the surface.

For dry mass (from now on DW) animals were put on pre-weighted aluminium foils, after that they were dried in stove (ST-01/02, Instrumentaria, Zagreb) for 24 hours at 70 °C. Dry mass was determined right after taking them out of the dessicator on a balance and measured to the nearest 0.1 mg.

With starting experiments we tested influence of WW / DW ratio on physiological response of animals of different size and/or age.

2.3.2 Microrespirometry

Measurements with Twin-Flow microrespirometer

Animals were held without food in synthetic water prior to the experiment for at least 24 h to avoid excretion in the experimental tube as well as to reduce oxygen consumption due to food digestion. Before animals were put in the test chamber they were rinsed with synthetic water. To reduce stress they were placed in the chamber at least three hours before measurements and to reduce movements of animals interior of the test chamber was kept dark during the measurement.

As a control of a system, the change in oxygen concentration without animal was measured every time. Average of this measurement was subtracted from measured average with animal.

All measurements of respiration with both systems were done in water reservoir with thermostat device that kept temperature at constant level with accuracy of 0.1 °C (see set-up in **Figure 12**).

Cyclobios microrespirometer was used in experiments with temperature and exposure experiments of *A. aquaticus* to copper sulfate and *G. fossarum* to imidacloprid. After that for measuring effects on *A. aquaticus* after exposure to imidacloprid, improved version of microrespirometer was tested and introduced. For all further experiments of exposure to atrazine, oxygen consumption was measured on new equipment.

2.3.3 Measuring ETS activity

All ETS activity measurements were made at 10 °C following the ETS protocol.

Solutions preparation

Homogenizing buffer, substrate solution, reagent solution (INT) and "stop" solution, were prepared on references of G.-Tóth (1993) (**Table 6**). Solutions were prepared just before analyses in order to avoid substrate decomposition and contamination with bacteria. Solutions were stored prior use on ice or in the refrigerator.

*Table 6: ETS solutions (*after *G.-Tóth, 1993)*

Homogenizing buffer

$MgSO_4$ (Sigma)	75 M
Polyvynylpyrrolidone PVP (Sigma)	0.15 % (w/v)
Triton-X-100 (Fluka)	0.2 % (w/v)
Solution was prepared in 0.1 M phosphate buffer, pH = 8.4.	

Substrate solution

NADH (Sigma))	1.7 mM
NADPH (Sigma)	0.25 mM
Triton-X-100 (Fluka)	0.2 % (w/v)
Solution was prepared in 0.1 M phosphate buffer, pH = 8.4.	

Reagent solution

Iodonitrotetrazolium chloride – INT (Fluka)	20 mg per 10 cm^{-3} of bi-distilled H_2O

"Stop" solution

Ortho-phosphoric acid - H_3PO_4 (85%; Merck): Formaldehyde (min 37%; Merck) = 1 : 1

Homogenate preparation

Animals were rinsed with distilled water and gently pressed against a paper towel in order to reduce excess water from their surface to the same degree for all animals. Fresh mass was determined on a balance to the nearest 0.1 mg. Afterwards they were homogenized in 4 ml ice cold buffer for 1.5 minutes with 600 rpm and followed by 20 sec homogenization with ultrasound (Cole-Parmer Instrument Co., 4710 series) at 40 W. Homogenates were centrifuged for 4 minutes at 0^{o}C and 10000 rpm.

Incubation

0.5 ml of the supernatant was incubated with 1.5 ml of substrate solution and 0.5 ml of INT solution at 10^{o}C. Every sample was incubated in triplicate. Control solutions were incubated without homogenate under the same conditions. The reaction was stopped after 40 minutes with "stop" solution. To all controls 0.5 ml of homogenate was added after "stop" solution.

Determination of formazane production and calculation of ETS activity

Formazane production was measured with spectrophotometer (WTW PhotoLab Spektral) at 490 nm wave length against control mix where homogenate was added after "stop" solution. All measurements were done within 10 minutes after stopping of reaction.
ETS activity was measured spectrophotometrically as the rate of reduction of tetrazolium to formazane and converted to equivalents of oxygen (in µl) utilized per wet mass per hour.

2.3.4 Measuring of total protein content in animals - BCA protein assay

A content of one albumin standard (BSA) ampoule was diluted into several clean vials using the same diluent as for samples. Each 1 ml of ampoule of 2.0 mg ml^{-1} albumin standard was used to prepare a set of diluted standards for determination of working range concentrations of proteins. There was sufficient volume for preparing three replications of each diluted standard as indicated in the **Table 7**.

Table 7: Preparation of diluted albumin (BSA) standards

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA concentration
A	700 µl	100 µl of Stock	250 µg ml^{-1}
B	400 µl	400 µl of vial A dilution	125 µg ml^{-1}
C	450 µl	300 µl of vial B dilution	50 µg ml^{-1}
D	400 µl	400 µl of vial C dilution	25 µg ml^{-1}
E	400 µl	100 µl of vial D dilution	5 µg ml^{-1}
F	400 µl	0	0 µg ml^{-1} = blank

Dilution scheme for Enhanced test tube protocol (working range = 5-250 µg ml^{-1})

Preparation of the BCA working reagent (WR)

Determination of total volume required:
(number of standards + number of unknowns) x (# replicates) x (volume of WR per sample) = WR total
Preparation of WR: mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (volume ratio 50 : 1, reagent A : B).

Test tube procedure was done in seven steps:

- 1. 0.1 ml of each standard and unknown sample replicate was transferred into an appropriately labeled test tube.
- 2. 2.0 ml of the WR was added to each tube and mixed
- 3. Tubes were covered and incubated in warm room or to thermo block (WTW CR 3200) at temperature 37^{o}C for 30 minutes.
- 4. Afterwards all tubes were cooled to room temperature
- 5. Spectrophotometer was set to 562 nm, instrument was zeroed with cuvette filled with distilled water. Absorbance was measured on all samples within 10 minutes.
- 6. The average 562 nm absorbance measurement of the blank standard replicate was subtracted from the 562 nm absorbance measurements of all other individual standards and unknown sample replicates.
- 7. Standard curve was prepared by plotting the average blank-corrected 562 nm measurement for each BSA standard *vs*. its concentration in µg ml^{-1}. The standard curve was used to determine the protein concentration of each unknown sample.

This curve was used for several assays of unknown samples; in practice it was repeatedly prepared every four months.

2.3.5 Calorimetric measurements

Calorimetric measurements were done on *G. fossarum* brought from Slovenia. Animal culture was held in mixed filtered water from animal collection site and synthetically prepared water in ratio 50 : 50. Animals were held at constant temperature around 15 °C with day/night regime 12/12 hours. Leaves from *Alnus glutinosa* were used for food and shelter for animals. At least one day before animals were put into the calorimeter they were brought to the lab from culture room. There they were held in water from culture which water and it was additionally aerated to maximal oxygen value with an air pump. At the same time the temperature was slowly raised to the room temperature.

Measurement method

Animals after one day without food were put into containers together with 750 µl of filtered and oxygen saturated water (by means of CFC filter). Water at room temperature was filtered just few minutes before use.

Containers were sealed with lids and mounted into calorimeter for 90 minutes. Data acquisition was programmed and initiated after 40 minutes; the time delay was to ensure that measured heat-flow was from the metabolism of the animal in resting state (only basic metabolism).

After measuring “basic” metabolism they were exposed for 1 hour to esfenvalerate solutions with different sublethal concentrations. Another measurement in calorimeter for each animal followed the exposures

following the same protocol as for "resting" state. All isotherm measurements in calorimeter were conducted at 15 °C.

After calorimetric measurements animals were rinsed with water and gently pressed against a paper towel in order to remove water from their surface to the same level at all animals. Fresh mass was determined to the nearest 0.1 mg.

Each individual was homogenized in 1.5ml ice cold 0.9% aqueous solution of NaCl with a Brown homogenizer (Ultra-Turrax-T8). Homogenate was centrifuged (Sigma 113) for 4 minutes at 6000 rpm and 1 ml of supernatant was stored in the freezer at -20 °C until it was used for measurements of enzymatic activity and the total protein content. They were de-frozen just before each measurement.

2.3.6 Stress enzymes

Stress enzyme measurements were done in triplicates according to protocols in the **Table 7** and **8**.

Table 8: GST test with CDNB

For each individual measurement this reagent mixture was prepared:	
0.1M phosphate buffer	675 µl
GRO – reduced glutathione	50 µl
Homogenate	12.5 µl
CDNB	12.5 µl
Total volume	750 µl

Reagents preparation

0.1M Phosphate buffer pH 8
7.0980 g Na_2HPO_4 (141.96 g mol^{-1}) / 500 ml dH_2O
6.8995 g $NaH_2PO_4xH_2O$ (137 g mol^{-1}) / 500 ml dH_2O

GR – Reduced Glutathione (15mM)
23 mg GSHred with 5 ml of 0.1M phosphate buffer

Substrate CDNB - 1-chloro-2,4-dinitrobenzen solution (72mM)
29.33 mg CDNB with 2 ml 96% EtOH

Standard
16.5 U / ml GSH – transferase from human placenta (G - 8642) E.C.2.5.1.18

Table 9: GPOx test with H_2O_2

For each individual measurement this reagent mixture was prepared:	
GSH – Reduced glutathione	700 µl
NADPH	25 µl
GR - glutathione reductase	9 µl
Homogenate (1 animal/ml 0.9% NaCl)	23 µl
H_2O_2 – 3 ‰ solution	50 µl
Total volume	807 µl

Reagents preparation

0.05 M Phosphate buffer with 1mM EDTA pH 7.9

GSH – Reduced glutathione 5.7mM

NADPH (4.3mM)

GR – Glutathione reductase 80mU

Substrate – 3 ‰ aqueous solution H_2O_2

Standard blind:	**Standard 2.5 U/ml:**
20 mg albumin	15 ml standard blind
46 mg reduced glutathione	375 µl (100 U/ml) GPOx from bovine
30 ml buffer	erythrocytes EC.1.11.1.9

Measurements were made by means of spectrophotometer Lambda 11 at 340 nm – each sample was measured every 30 s in an interval of 6 minutes.

2.4 Data processing

2.4.1 Calculations

R data from Cyclobios microrespirometer were calculated in DatGraph 2.1 Analysis (Oroboros®) to access values of oxygen consumption. Concentration of oxygen was gained from the table of oxygen solubility at different temperatures and atmospheric pressure (Gnaiger and Forstner, 1983).

ETS activity was calculated by equation:

ETS activity (µl O_2 $S^{-1}h^{-1}$) = ($Abs^{490\ nm}$ * V_r * V_h * 60) / (V_a * S * t * 1.42)

$Abs^{490\ nm}$	absorption of sample
V_r	final reaction volume (3 ml)
V_h	volume of homogenate (4 ml)
V_a	volume of incubated homogenate (0.5 ml)
S	sample size (mg of wet mass)
T	incubation time (min)
1.42	factor of conversion to oxygen volume (Kenner and Ahmed, 1975a)

Flow of water was measured directly and oxygen consumption was calculated by formula:

Oxygen consumption (mg h^{-1}) = c (at inflow) – c (at outflow) x flow per hour;

where “c” means oxygen concentration in mg l^{-1}.

Later after changing the measuring method, R data were acquired from the Data file from OXY-4 (PreSens). Data were imported in Windows Excel 2002 and mean values were calculated from graphs.
At the start of using microcalorimeter, calibration procedure was done and data were collected and conversed to heat capacity directly one the DSC. Data files were than imported and analyzed in Microsoft excel. Data of heat emission differences from microcalorimetric measurements (MC-DSC Calorimetry Science Corp., Lindon, UT) were imported in Windows Excel 2002. Values were calculated from the graphs - the difference between means of calorimetric measurement curves before and after treatment with esfenvalerate were deducted from a baseline curve taken from isotherm measurement at 15^{o}C. Calculation was based on differences in heat between reference chamber which was always empty and other one filled with water and animal.

Data for stress enzyme measurement were calculated in Windows Excel 2002 from standard curve; which was acquired and tested before stress enzyme activity measurements with enzyme standard.

Acquired data from microcalorimetric and from stress enzyme measurements had high differences and for easier presentation they were ln transformed.

2.4.2 Statistical analyses

Windows Excel 2002 was used for sorting and calculation of rough data from all experiments. All data sets were analyzed in computer program SigmaStat 3.5 (SYSTAT). For ETS/R ratios at the start basic descriptive statistics were extracted, if normality test and equal variance test passed, simple ANOVA or MANOVA was performed. In the case if a normal distribution failed, nonparametric tests on ranks were used, like Kruskal-Wallis One Way Analysis of Variance or Mann-Whitney Rank Sum Test. Additional test (Dunn's Method and Holm-Sidak method) were performed in order to establish difference between some groups, which were on the margin of significance.

For each figure in the results data were compared against control and against each other. Results of pair-wise comparisons are indicated below each graph; different letter below data group means groups are significantly different.

3. RESULTS

3.1 Incidence of wet (WW) and dry mass (DW) ratio

Ratio between wet mass (WW) and dry mass (DW) shows difference between water content in tested animals. Results for *A. aquaticus* are presented in the **Figure 15** and for *G. fossarum* in the **Figure 16**.

The ratio between WW and DW for both test animals is more constant in large animals than in smaller ones. In smaller individuals of *A. aquaticus*, i.e. animal mass < 20 mg, the ratio between WW and DW is on an average 4.9 ± 1.0 mg (n=9). In specimens with WW from 20 mg to 45 mg the ratio is significantly higher with an average 6.7 ± 1.8 mg (n= 11) (t-test t= 2.721; d.f.= 19; $P<0.05$).

The picture is almost the same in case of *G. fossarum.* In smaller individuals, i.e. animal WW < 20 mg, the ratio between WW and DW is on an average 3.9 ± 0.5 mg (n=13). In specimens with WW from 20 mg to 45 mg the ratio is significantly higher with an average 4.8 ± 0.6 mg (n=11) (Mann-Whitney; T= 172; $P<0.05$). With increasing size of the animals, water content in animal tissues is also getting higher. Regression lines and equations for both test species are compared in the **Figure 17**.

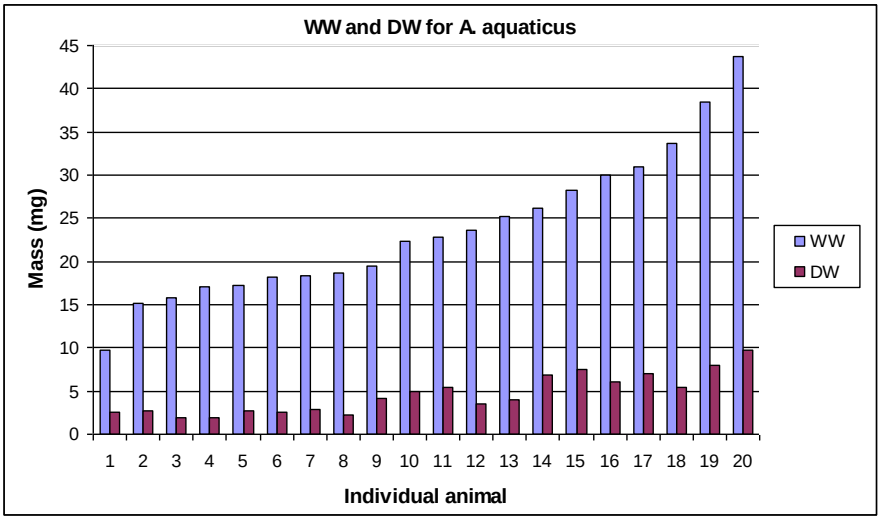

***Figure 15**: Fresh and dry mass for* Asellus aquaticus*. WW – wet mass of individual animal in milligrams; DW – dry mass of individual animal in milligrams after 24 hours at 70 °C (n = 20 individuals).*

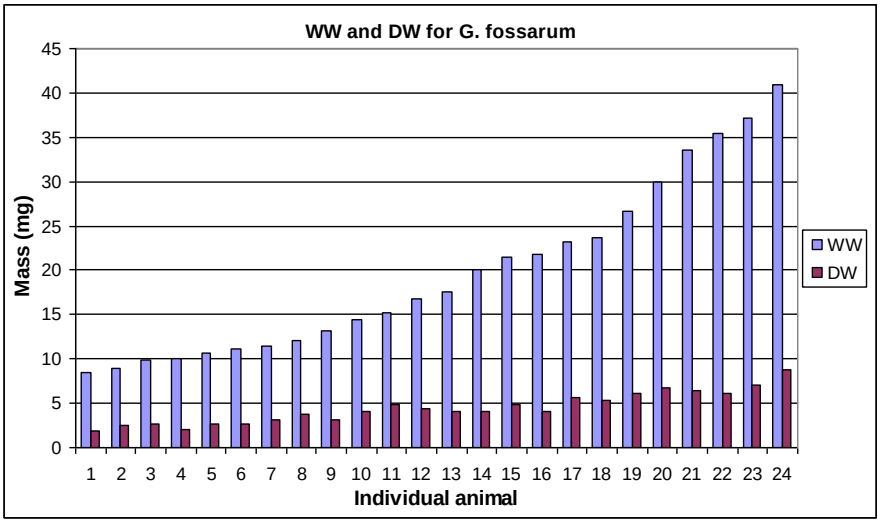

***Figure 16**: Fresh and dry mass for* Gammarus fossarum*. WW – wet mass of individual animal in milligrams; DW – dry mass of individual animal in milligrams after 24 hours at 70 °C (n = 24 individuals).*

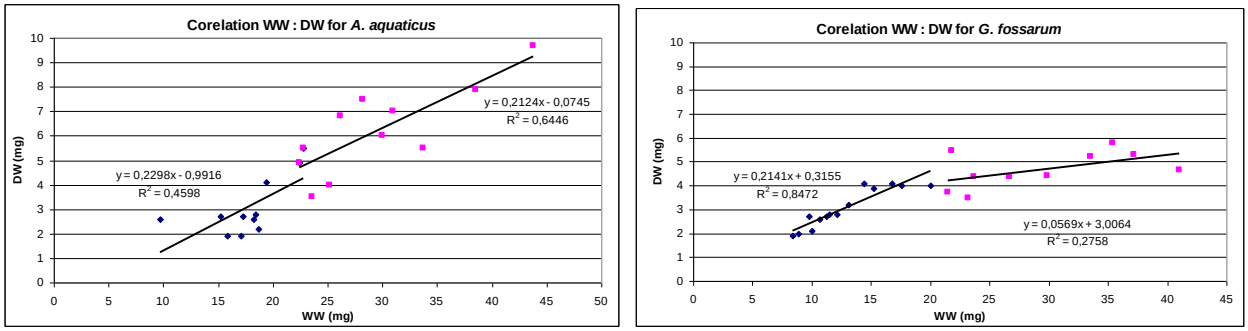

***Figure 17**: Regression between wet and dry mass for* Asellus aquaticus *and* Gammarus fossarum. *WW – wet mass of individual animal in milligrams; DW – dry mass of individual animal in milligrams after 24 hours at 70 °C (n = 20 individuals).*

3.2 Effects of temperature on respiration and ETS/R ratio

Asellus aquaticus from main location the Lake Cerkniško jezero and alternative location the Iščica River

Results for animals **WW** and respiration measurements are in the **Table 10**. Animal population collected from the Iščica River consisted from smaller individuals (lower **WW**) than animals collected from the lake Cerkniško jezero (main location). Culture of animals from the Lake Cerkniško jezero maintained at 10 °C was used as a control group (**blue color**). In the first step they were compared to the group of animals from alternative location (i.e. Iščica River), also maintained at 10 °C. In the second step they were compared with a group of animals from the same location, but reared at 15 °C.

R rate in animals collected from the same location is significantly higher at 15 °C compared to 10 °C (t-test; t = 2.753; d.f. = 56; p<0.05). **ETS** activity values are statistically different at different temperatures from the same location (P<0.001). ETS values from two different locations at the same temperature are not different.

Table 10: Asellus aquaticus *measurements at two different temperatures; mean values ± SD*

	10 °C Iščica	SD	**10 °C Cerkniško**	SD	15 °C Cerkniško	SD
WW (mg)	**10.1*** **	2.4	**21.5**	7.5	**13.9****	1.9
R (µl O_2 mg^{-1} h^{-1})	**0.08***	0.02	**0.06**	0.02	**0.10***	0,04
ETS (µl O_2 mg^{-1} h^{-1})	**0.2892**	0.0523	**0.3271**	0.0908	**0.7437*** **	0.1543

* *P < 0.05*
** *P < 0.01*
*** *P< 0.001*

Gammarus fossarum from the main location - Iščica River and alternative location - the spring at Duplje cave

Results for animals **WW** and respiration measurements are in the **Table 11.** Animals from the Iščica River have similar average **WW,** while from the spring near village Duplje it is significantly lower. Culture of animals from the Iščica River maintained at 10 °C were used as a control group (**blue color**) and compared to group of animals from another location (Duplje) and also maintained at 10 °C. In the second comparison were to groups of animals from same location (Iščica) but maintained at 15 °C.

R rate at the group from Iščica maintained at 15 °C was significantly higher than at control group and same significant difference was shown also at group of animals from the spring at Duplje (p<0.001). **ETS** activity did not differ at both tested temperatures nor at animals from both locations.

Table 11: Gammarus fossarum *measurements at two different temperatures; mean values ± SD*

	10 °C Duplje	SD	**10 °C Iščica**	SD	15 °C Iščica	SD
WW (mg)	**15,9*** **	4,2	**27,5**	7,2	**29,8**	12,6
R (µl O_2 mg^{-1} h^{-1})	**0,09*** **	0,02	**0,16**	0,05	**0,10*** **	0,02
ETS (µl O_2 mg^{-1} h^{-1})	**0,4077**	0,0964	**0,3794**	0,0915	**0,3730**	0,1216

*** *P< 0.001*

Results of **ETS/R ratio** are in **Figure 18**. Individuals of *A. aquaticus* from two different locations have significantly different ETS/R ratio at 10 °C (Kruskal-Wallis analysis of ranks; H = 41.839 with 2 d. f.; P<0.05)) ETS/R ratio is also significantly higher at 15 °C than at 10 °C in individuals from the main location – Zadnji kraj (the Lake Cerkniško jezero) (p<0.001). **ETS/R** ratio for *G. fossarum* is significantly higher at animals from the alternative location - spring near Duplje at 10 °C (F = 22.406; d.f.= 2; p<0.001) and higher at animals from the main location – the Iščica River at 15 °C (t-test; t = 3,659; d.f.= 39; p<0.001).

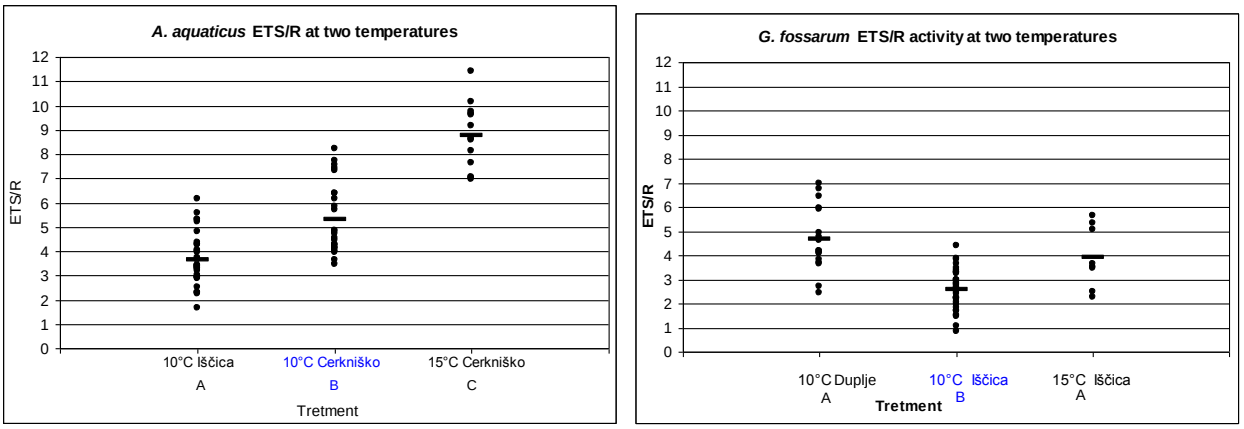

Figure 18*: The effect of temperature on ETS/R ratio for* A. aquaticus *and* G. fossarum *from two different locations. Dots are the results from the individual animals; line is the mean of the group.* ***The first column:*** *measurements at 10 °C from alternative location;* ***the second column:*** *measurements at 10 °C from the main location;* ***the third column****: measurements at 15 °C from the same location. (n= 13; 25; 32 individuals of* A. aquaticus *and n= 16; 33; 8 individuals* of G. fossarum*). Results of pair-wise comparisons are indicated below the graph; the different letter below data group means groups are significantly different (p<0.05).*

3.3 Toxicity tests results

Table 12: LC_{50} (48h) and EC_{50} (48h) for A. aquaticus *and* G. fossarum

	24 hour exposure	*A. aquaticus*	
Cu^{2+}	LC_{50} (48h)	30 mg l^{-1}	
	EC_{50} (48h)	20 mg l^{-1}	
	1 hour exposure	*A. aquaticus*	*G. fossarum*
imidacloprid	LC_{50} (48h)	10 mg l^{-1}	1 mg l^{-1}
	EC_{50} (48h)	1 mg l^{-1}	0.1 mg l^{-1}
	1 hour exposure	*A. aquaticus*	*G. fossarum*
atrazine	LC_{50} (48h)	30 mg l^{-1}	10 mg l^{-1}
	EC_{50} (48h)	0.1 mg l^{-1}	0.1 mg l^{-1}
	1 hour exposure		*G. fossarum*
esfenvalerate	LC_{50} (48h)		0.3 µg l^{-1}
	EC_{50} (48h)		0.02 µg l^{-1}

Copper sulfate used for 48 hour exposure solutions ranged from 1, 3, 10, 30, 100 mg l^{-1} of Cu^{2+} in aqueous medium. Experiments with this substance were conducted only on ***A. aquaticus***. Tested LC_{50} (48h) was 30 mg l^{-1} of Cu^{2+}, EC_{50} (48h) was 20 mg l^{-1} of Cu^{2+}. Chosen sublethal 24 hour exposure concentrations for further experiments were lower than 10 mg l^{-1} of Cu^{2+}.

Imidacloprid was tested on both test species ***A. aquaticus*** and ***G. fossarum***. For ***A. aquaticus*** LC_{50} (48h) was 10 mg l^{-1} and EC_{50} (24h) was 1 mg l^{-1} of imidacloprid. Selected 1 hour sublethal concentrations were 10 mg l^{-1} and lower. For ***G. fossarum*** established LC_{50} (48h) was 1 mg l^{-1}; while EC_{50} (24h) was 0.1 mg l^{-1} of imidacloprid. Sublethal concentrations for 1 hour exposure were same as for *A. aquaticus* (10 mg l^{-1} and lower).

Atrazine was also tested on both test species ***A. aquaticus*** and ***G. fossarum***. For ***A. aquaticus*** measured LC_{50} (48h) was 30 mg l^{-1} and EC_{50} (24h) was 0.1 mg l^{-1} of atrazine. For ***G. fossarum*** LC_{50} (48h) was 10 mg l^{-1} and EC_{50} (24h) was 0.1 mg l^{-1} of atrazine. One hour exposure concentrations used were equal or lower than 10 mg l^{-1}.

Esfenvalerate was tested on ***G. fossarum*** and LC_{50} (48h) was determined at 0.3 µg l^{-1}. One hour exposure concentrations used were 0.3 µg l^{-1} and lower.

3.4 Effects of chemicals on respiration

3.4.1 Exposure to copper sulfate solutions

Effects of exposure to copper sulfate on *A. aquaticus* are in the **Table 13**.

Randomly selected animals with similar wet mass were used for this test. Sample sizes were from 5 to 8 animals. Mean **WW** in different groups ranged from 17.8 to 21.9 mg.

There is no statistically significant difference in **R** between the groups of *A. aquaticus* exposed to different concentrations of Cu^{2+}. Average values of **R** at treated groups were similar as at untreated group and **ETS** activity values show inconsistency.

The difference in the mean values of **ETS/R** between any two groups is not great enough to reject the possibility that the difference is due to random sampling variability (**Figure 21**).

Table 13: Asellus aquaticus *exposed to copper sulfate; mean values ± SD*

	Group 1		Group 2		Group 3		Group 4	
	Control	SD	3 mg l^{-1}	SD	5 mg l^{-1}	SD	10 mg l^{-1}	SD
WW (mg)	**20.8**	4.6	**21.9**	6.9	**17.8**	4.2	**21.1**	2.1
R (µl O_2 mg^{-1} h^{-1})	**0.12**	0.01	**0.12**	0.04	**0.10**	0.04	**0.12**	0.03
ETS (µl O_2 mg^{-1} h^{-1})	**0.4798**	0.0897	**0.4090**	0.1016	**0.5429**	0.2440	**0.3965**	0.0422

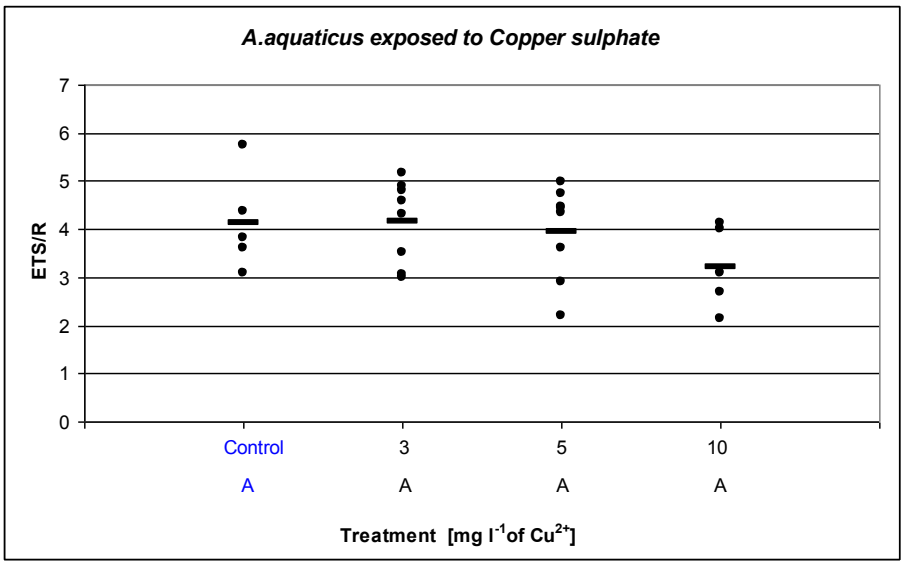

Figure 19; *ETS/R ratio for* Asellus aquaticus *exposed for one day to different copper sulfate sublethal solutions. Electron transport system (ETS) activity is expressed as related to wet mass. Dots are the results from individual animals; line is the mean of the group. Treatments are control and exposure concentrations of 3, 5, 10 mg l^{-1} Cu^{2+}, respectively; (n = 5; 8; 8; 5 individuals). Results of pair-wise comparisons are indicated below the graph; the same letter below data group means groups are not significantly different.*

3.4.2 Exposure to imidacloprid solutions

Results in the **Table 14** show animal selection in groups of *A. aquaticus* with **WW** averaging from 15.7 to 22.3 mg.

Measured **R** in groups 4 and 5 compared to Control group 1, is increased in average for around 20%; which is also significantly higher (P<0.005). **ETS** activity values from all groups were quite comparable; accept from group 5 where main value was significantly lower for c. 25% (P<0.001).

Table 14: Asellus aquaticus *exposed to imidacloprid; mean values ± SD*

	Group 1		Group 2		Group 3		Group 4		Group 5	
	Control	SD	0.01 mg l^{-1}	SD	0.1 mg l^{-1}	SD	1 mg l^{-1}	SD	10 mg l^{-1}	SD
WW (mg)	**15.7**	3.0	**21.8**	4.1	**16.9**	2.8	**22.3**	4.3	**19.7**	2.9
R (µl O_2 mg^{-1} h^{-1})	**0.14**	0.04	**0.15**	0.03	**0.13**	0.03	**0.19****	0.04	**0.17****	0.03
ETS (µl O_2 mg^{-1} h^{-1})	**0.2962**	0.0419	**0.2907**	0.0456	**0.2727**	0.0447	**0.2814**	0.0474	**0.2077*****	0.0311

** *P < 0.01*
*** *P< 0.001*

Results in the **Table 15** show that individuals of *G. fossarum* were randomly selected for this experiment with average **WW** ranging from 14.4 to 22.7 mg. Similar WW were in pairs; group 2 and 3 as well as group 1 and 4. Group sample size was from 11 (in group 1) and up to 25 animals (**Figure 20**).

There is resemblance between measured **R** in all four groups. **ETS** values from Group 4 show statistically significant difference from all other groups (Kruskal-Wallis; H= 19.721; d.f.= 3; P<0.05). Differences between those groups are greater than would be expected by chance.

Table 15: Gammarus fossarum *exposed to imidacloprid; mean values ± SD*

	Group 1		Group 2		Group 3		Group 4	
	Control	SD	0.01 mg l^{-1}	SD	0.1 mg l^{-1}	SD	10 mg l^{-1}	SD
WW (mg)	**14.4**	4.3	**22.3**	4.5	**22.7**	3.9	**15.8**	3.2
R (µl O_2 mg^{-1} h^{-1})	**0.09**	0.02	**0.13**	0.03	**0.10**	0.03	**0.11**	0.04
ETS (µl O_2 mg^{-1} h^{-1})	**0.3956**	0.1042	**0.3527**	0.0823	**0.3538**	0.0691	**0.2708****	0.0713

** *P < 0.01*

With increasing imidacloprid value in exposure solutions, statistical significant difference in mean **ETS/R** ratio for *A. aquaticus* is shown for 1 and 10 mg l^{-1} of imidacloprid. Kruskal-Wallis analysis of Variance on ranks is used since equal variance test of grouped data failed. ETS/R from groups exposed to 1 and 10 mg l^{-1} of imidacloprid show statistically significant difference between them and all other groups (Kruskal-Wallis analysis of ranks; H = 51.053; with d.f.= 4; P<0.05). Differences are greater than would be expected by chance (**Figure 20**).

Lower **ETS/R** ratio for *G. fossarum* in group exposed to 10 mg l^{-1} of imidacloprid is due to about 30% lower ETS value; which is significantly lower compared to Control group 1 (P<0.001). For increasing imidacloprid value in exposure solutions statistical significant difference of ETS/R is shown for 0.01 and 10 mg l^{-1} of imidacloprid. In group exposed to 0.1 mg l^{-1} of imidacloprid difference was not statistically different from Control (**Figure 20**).

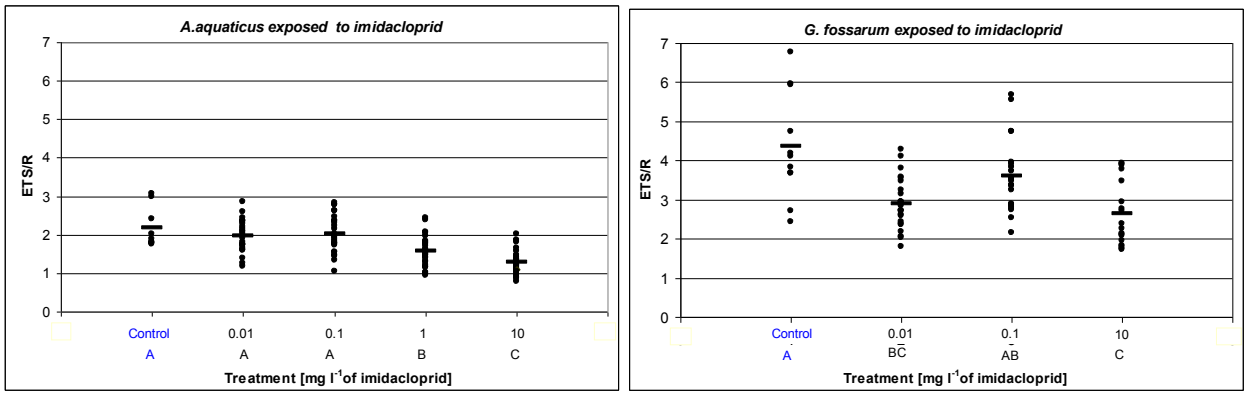

***Figure 20**; ETS/R ratio for* Asselus aquaticus *and* Gammarus fossarum *exposed for one hour to different imidacloprid sublethal solutions. Electron transport system (ETS) activity is expressed as related to wet mass. Dots are the results from individual animals; line is the mean of the group. On X line there are treatments with different concentrations of imidacloprid (left n = 9; 24; 30; 26; 30 individuals of* A. aquaticus and right *n = 11; 25; 21; 18 individuals of* G. fossarum*). Results of pair-wise comparisons are indicated below the graph; the different letter below data group means groups are significantly different (p<0.001).*

3.4.3 Exposure to atrazine solutions

Results for *A. aquaticus* are shown in the **Table 16. WW** from randomly selected animals was quite constant; in average from 9.9 to 13.7 mg. Exposure group size was from 9 to 10 animals. Results from total protein content (PROT) in Control group 1 and group 3 (10 mg l^{-1} of atrazine) are similar, while in group 2 (5 mg l^{-1} of atrazine) the value is significantly higher (p<0.05).

Variance in **R** was not equal; in group 3 (10 mg l^{-1} of atrazine), the mean R is increased in average for around 60%. Which is more than would be expected by chance; it is significantly different (P<0.001). Mean values of **ETS** activity among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; the differences are not significant.

Table 16: Asellus aquaticus *exposed to atrazine; mean values ± SD*

	Group 1		Group 2		Group 3	
	Control	SD	5 mg l^{-1}	SD	10 mg l^{-1}	SD
WW (mg)	**11.8**	3.3	**9.9**	3.4	**13.7**	3.9
PROT (mg/WW)	**0.320**	0.116	**0.505**	0.087	**0.369**	0.131
R (µl O_2 mg^{-1} h^{-1})	**0.054**	0.016	**0.056**	0.021	**0.133*** **	0.034
ETS (µl O_2 mg^{-1} h^{-1})	**0.478**	0.096	**0.468**	0.060	**0.526**	0.063

*** *P< 0.001*

Results for *G. fossarum* are presented in the **Table 17.** Mean **WW** of *G. fossarum* are very consistent ranging from 14.1 up to 16.2 mg. Group size is from 18 to 22 animals. Protein content values in all groups are similar.

Variance in **R** was not equal; in group 3 (3 mg l^{-1} of atrazine) and 4 (10 mg l^{-1} of atrazine) mean R is increased in average for around 30%. Which is more than would be expected by chance; there is a statistically significant difference (P< 0.005). In group 2 (1 mg l^{-1} of atrazine), R is increased for 17% but this raise is not significant (P = 0.118). Mean values of **ETS** activity among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is insignificant difference (P= 0.087).

Table 17: Gammarus fossarum *exposed to atrazine; mean values ± SD*

	Group 1		Group 2		Group 3		Group 4	
	Control	SD	1 mg l^{-1}	SD	3 mg l^{-1}	SD	10 mg l^{-1}	SD
WW (mg)	**16.3**	3.5	**14.8**	3.4	**16.2**	3.8	**14.1**	4.2
PROT (mg/WW)	**0.511**	0.084	**0.450**	0.161	**0.503**	0.081	**0.579**	0.103
R (µl O_2 mg^{-1} h^{-1})	**0.074**	0.018	**0.087**	0.024	**0.097****	0.032	**0.094****	0.018
ETS (µl O_2 mg^{-1} h^{-1})	**0.478**	0.065	**0.424**	0.094	**0.435**	0.073	**0.458**	0.092

** *P < 0.01*

Figure 21 shows **ETS/R** ratio results for both test species. Statisticaly significant difference in **ETS/R** ratio for *A. aquaticus* is shown for group 3 (10 mg l^{-1} of atrazine) and also group 2 (1 mg l^{-1} of atrazine) when compared to Control group 1. Equal variance test of grouped data passed and one way ANOVA statistics is used. ETS/R ratio values from Group 3 (10 mg l^{-1} of atrazine) show statistically significant difference compared to Control group 1 and also from group 2 (ANOVA; F= 13.914; d.f.= 2; P<0.001). Differences between those groups are greater than would be expected by chance.

For increasing atrazine value in exposure solutions for *G. fossarum*, statistical significant difference is shown for groups 2, 3 and 4 (1, 3 and 10 mg l^{-1} of atrazine). **ETS/R** ratio values from all tested groups, 2 (10 mg l^{-1} of atrazine), 3 (3 mg l^{-1} of atrazine) and 4 (5 mg l^{-1} of atrazine) show statistically significant difference compared to Control group 1 (P<0.05). Differences among the groups are greater than would be expected by chance; there is a statistically significant difference (ANOVA; F= 10.549; d.f.= 3; P<0.001).

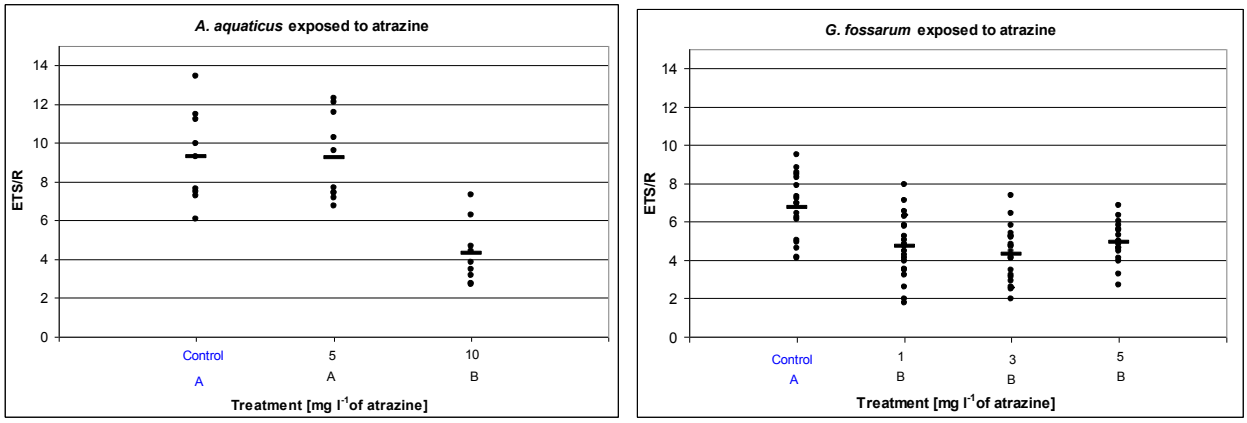

Figure 21*; ETS/R ratio for* Asellus aquaticus *and* Gammarus fossarum *exposed for one hour to different atrazine solutions. Electron transport system (ETS) activity is expressed as related to wet mass. Dots are the results from individual animals; line is mean of the group. X line are treatment with different concentrations of atrazine; (left n = 9; 10; 9 individuals of* A. aquaticus and on the right *n = 18; 21; 22; 18 individuals of* G. fossarum*). Results of pair-wise comparisons are indicated below the graph; the different letter below data group means groups are significantly different (p<0.001).*

3.5 Effects of chemicals on metabolic rate

3.5.1 Calorimetric measurements on *Gammarus fossarum*

Table 18: Gammarus fossarum *exposed to esfenvalerate; mean values ± SD*

	Group 1		Group 2		Group 3		Group 4		Group 5	
	Control	SD	**0.02 µg l^{-1}**	SD	0.1 µg l^{-1}	SD	0.2 µg l^{-1}	SD	0.3 µg l^{-1}	SD
WW (mg)	**9.2**	3.5	**9.2**	3.1	**8.4**	1.7	**8.9**	4.0	**9.5**	1.4
DW (mg)	**2.0**	0.8	**2.1**	0.7	**1.9**	0.4	**2.0**	0.9	**2.2**	0.8
PROT (mg)	**1.5**	0.5	**1.5**	0.5	**1.1**	0.4	**1.4**	0.2	**1.4**	0.4
ΔHEAT (µW)	**0.962**	0.373	**0.990**	0.146	**2.293**	0.955	**6.513**	5.885	**137.564**	133.305
ln ΔHEAT	**0.656**	0.197	**0.685**	0.074	**1.155**	0.276	**1.796**	0.642	**4.328**	1.219

Measured differences in heat production of animals before and after treatment with esfenvalerate are shown in the **Figure 22.** Mean **WW** of animals in different groups are very uniform. Treatment groups in this experiment are from 13 to 16 animals.

Means of different concentration of esfenvalerate exposures are tested and statistics show significant effect (Kruskal-Wallis analysis of ranks F= 86.492; d.f.= 4; P<0.001). There is no effect on heat measurements in group 1 (0.02 µg l^{-1}), but in all other groups there are significantly important differences compared to control.

In the **Figure 22** data of heat differences measured with microrespirometer are expressed as logarithmic values. The same results were obtained when values were calculated in regard to protein content from each animal or in regard to dry mass of animal. Both results show significant difference.

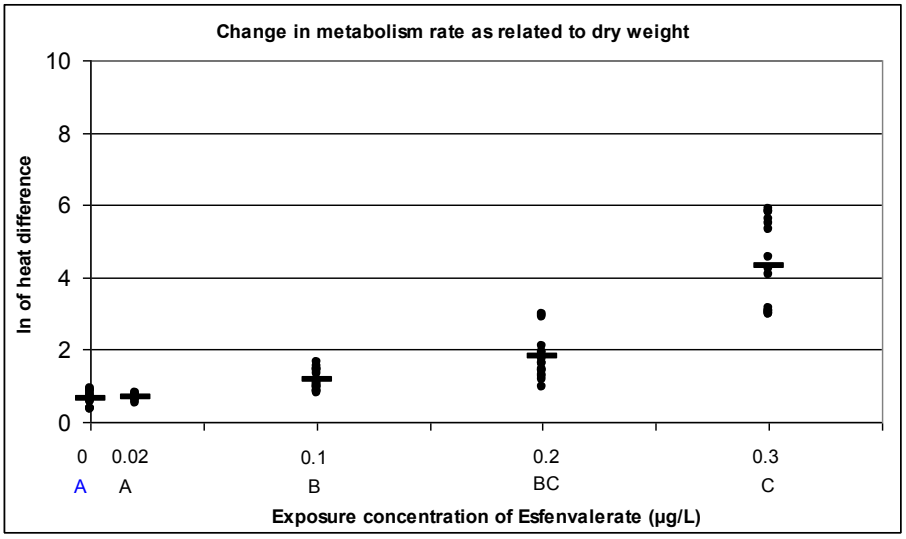

Figure 22*; Change in metabolism rate for* Gammarus fossarum *after exposure for one hour to different esfenvalerate sublethal solutions. Heat difference is expressed in µW and ln transformed. Dots are the results from individual animals and were calculated regarding to dry mass (lnDW) of animals; line is mean of the group. Treatment concentrations were; control (as 0), 0.02, 0.1, 0.2 and 0.3 µg l^{-1} of esfenvalerate; (n = 13; 13; 16; 16; 15 individuals). Results of pair-wise comparisons are indicated below the graph; the different letter below data group means groups are significantly different (p<0.05).*

3.5.2 Stress enzyme measurements on *Gammarus fossarum*

Sample size for all enzyme testing was from 13 to 18 animals. Stress enzyme measurement results are presented in the **Table 19**.

GST activity after one hour exposure of animals to different concentrations of esfenvalerate did not change; differences observed were not significant (**Figure 23**). Although enzyme activity is higher for 40% at concentration 0.3 µg l^{-1}, it is not significantly different. Variance is not equal and statistical test does not show the difference.

GPOx activity is significantly affected by one hour esfenvalerate exposure (**Figure 24**). Animals exposed to concentrations greater than 0.1 µg l^{-1} of esfenvalerate show elevated level of GPOx activity (ANOVA; F=37.258; d.f.= 4; P<0.001). Mean values are up for 220%; from 16.7 (Control) up to 54 nmol min^{-1} mg^{-1} of protein (group 0.3 µg l^{-1}).

Table 19: Gammarus fossarum *exposed to esfenvalerate; GST and GPOx activity; mean values (nmol min^{-1} mg^{-1} protein) ± SD*

	Group 1		Group 2		Group 3		Group 4		Group 5	
	Control	SD	0.02 µg l^{-1}	SD	0.01 µg l^{-1}	SD	0.2 µg l^{-1}	SD	0.3 µg l^{-1}	SD
GST	**3399.03**	1279.04	**3887.13**	1386.41	**3857.13**	1347.85	**3883.55**	1011.72	**4797.59**	1860.69
lnGST	**8.046**	0.464	**8.186**	0.450	**8.211**	0.298	**8.230**	0.275	**8.406**	0.388
	Control	SD	0.02 µg l^{-1}	SD	0.01 µg l^{-1}	SD	0.2 µg l^{-1}	SD	0.3 µg l^{-1}	SD
GPOx	**16.706**	6.950	**15.249**	6.153	**35.523**	8.931	**42.748**	14.356	**54.005**	24.395
lnGPOx	**2.718**	0.488	**2.641**	0.435	**3.541**	0.249	**3.709**	0.306	**3.904**	0.423

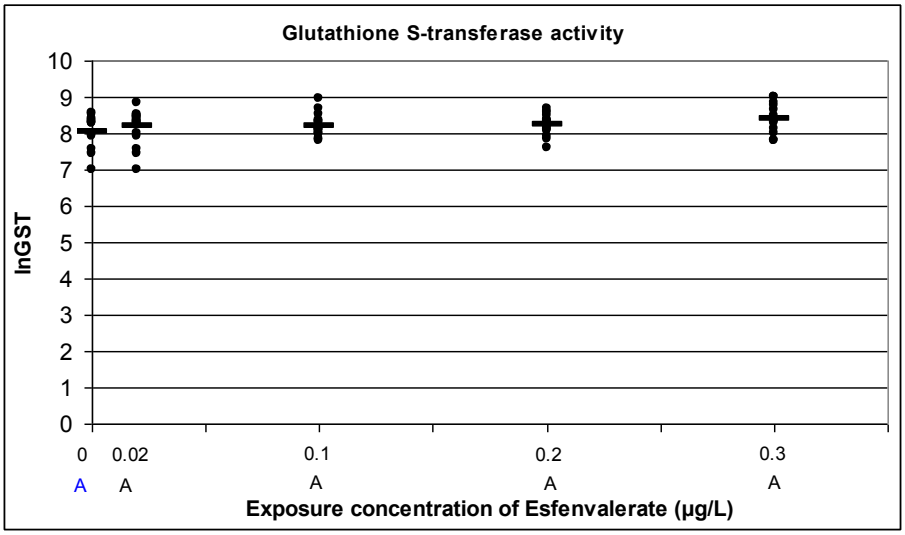

***Figure 23**; Enzyme glutathione transferase (GST) activity from* Gammarus fossarum *after exposure for one hour to different esfenvalerate sublethal solutions. Enzyme activity is expressed in nmol min^{-1} mg^{-1} protein and than ln transformed. Dots are the results from individual animals; line is mean of the group. Treatment concentrations were; control (as 0), 0.02, 0.1, 0.2 and 0.3 µg l^{-1} of esfenvalerate; (n = 13; 18; 18; 18; 15 individuals). Data were calculated regarding to total protein content of animals. Results of pair-wise comparisons are indicated below the graph; the same letter below data group means groups are not significantly different.*

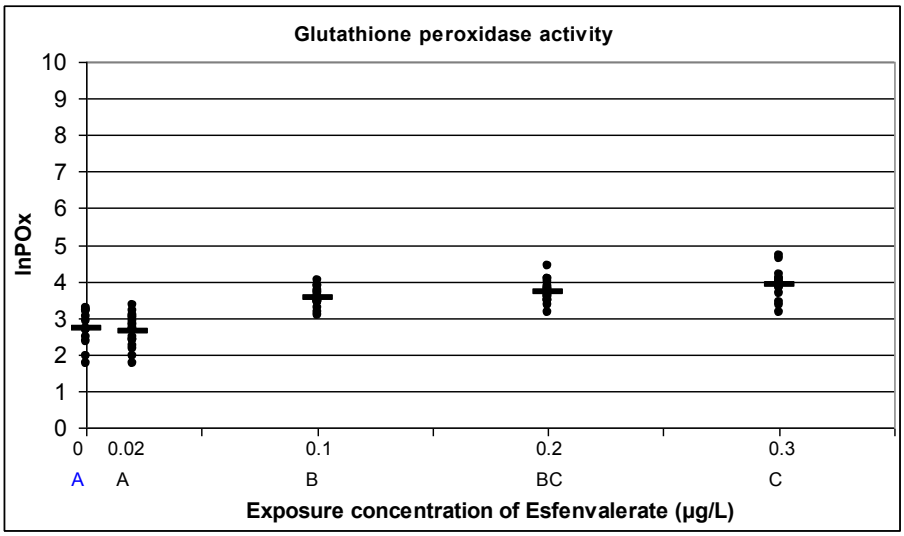

Figure 24; *Enzyme glutathione peroxidase (GPOx) activity from* Gammarus fossarum *after exposure for one hour to different esfenvalerate sublethal solutions. Treatment concentrations were; control (as 0), 0.02, 0.1, 0.2 and 0.3 µg* l^{-1} *of esfenvalerate; (n = 13; 18; 18; 18; 15 individuals). Enzyme activity is expressed in nmol* min^{-1} mg^{-1} *protein and than ln transformed. Dots are the results from individual animals; line is mean of the group. Data were calculated regarding to total protein content of animals. Results of pair-wise comparisons are indicated below the graph; the different letter below data group means groups are significantly different (p<0.05).*

4. DISCUSSION

4.1 Importance of this work

In first part of the work ETS and the R measurements were conducted. For ecotoxicological studies application of those two methods together was the first application ever of this stress estimation method. All other authors presented toxic effects in some other way, mainly with toxicity tests alone i.e. LC_{50} or EC_{50} the latter based primarily on locomotion effects only.

Both methods together are good predictors of the stress state of test animals. ETS activity represents potential maximum uptake of oxygen, while respiration represents realized oxygen consumption. That for, theoretically, ETS/R ratio around 1 means that R activity is running at the 100% capacity and that is indication that animal is close to its physiological limits (Bamstedt, 1980).

In the second part the calorimetric measurements show potential for use in ecotoxicology as well as measurement of stress enzyme activity. Both methods together are a good predictor of stress. Since animals in stress spend and release more energy that can be measured on microcalorimeter. Stress enzyme activity can easily be measured spectrophotometrically.

All chosen methods could be integrated into ecotoxicological studies. Stark and Banks (2001) are pointing out that new selective insecticides should be tested also on non-target species that can get into contact with applied pesticide.

4.2 Comparison of methods used in this work

4.2.2 Test animals

Test species were selected according to reports from other authors about their reasonable susceptibility to different pollutants. Test species used in laboratory represent only a tiny part of natural aquatic fauna and response variability is thus rather narrow. From this point of view use of other animals in toxicity tests should be reconsidered (Cairns and Niederlehner, 1987). *Daphnia magna* is a preferred test species in aquatic toxicology but it has limited responses due to parthenogenesis. Other animals that do not exhibit parthenogenesis can have much more diverse responses to certain stress factor (Fernandez-Alba et al., 2002). *Daphnia magna* also exists only in some temperate Palearctic lakes and does not occur in the lotic environment (McCahon and Pascoe, 1988). Experiments that are conducted in running freshwater systems have different ecological specifics than those from still water. The water current can have additional effect on test animals so it is necessary to include it in the term “controlled conditions”. Test animals used in our experiments were collected from sites with different water current. *A. aquaticus* dominates in freshwaters with slower current while *G. fossarum* colonizes areas with faster water currents (Naylor et al. 1990; Graça et al., 1994a).

In four years period we tested mainly *A. aquaticus* collected from Zadnji kraj at the Lake Cerkniško Jezero with rather limited rheocene habitat. Only at the beginning of experiments *A. aquaticus* was also collected from the Iščica River. Another species that was tested is *G. fossarum* location was at first from the Iščica River and later from Cave Dupeljska jama entrance. Change of location from original site the Iščica River, was due to unexpected reconstruction works and high water floods in autumn 2006. That caused complete removal of sediment along the river bed in the Iščica River, resulting in reduction of local population of *G. fossarum* at selected collection site. However both *G. fossarum* populations inhabit fast-flowing and well-oxygenated reaches of streams.

Other authors also used *A. aquaticus* for a comparison to *G. pulex* as a model relation. Both species exhibit differences in locomotion, respiration and mostly in susceptibility to some toxicants, like zinc and ammonia (Naylor et al., 1990; Rinderhagen et al., 2000). Both species are suitable for stress measurements of toxicants coming to freshwater environment i.e. as non-target species affected by pesticides.

Animals must be tested for parasites by means of dissection before lab culture was established (Pascoe et al., 1995). Parasitism could have influence not only on stabile population of laboratory culture but also on their physiology, incl. basal metabolism. In collected population from the nature, infected specimens of *A. aquaticus* can be easily spotted and removed from the laboratory stock. Dark pigmentation of animals indicates that acantocephalan parasite is present (Cezilly et al., 2000).

4.2.2 Size of animals – WW and DW

Bamstedt (1980) defined that ETS activity and R are related to WW of the animal. King and Packard (1975) established that influence of body size on ETS and respiration is mathematically equivalent, since both processes are connected. Musko et al. (1995) ascertained that size of animals had no influence on ETS/R ratio. Simčič and Brancelj (2000) reported that ETS decreased with increasing size in *Chirocephalus croaticus*. All those statements are not in accordance and they confirmed our aim to have animals of the same size and mass in individual experiments. From the first experiments we figured that size of an animal does have an influence. Smaller animals have higher ETS values than larger ones and vice versa. WW against DW ratio showed that larger/older animals have higher amount of metabolically inactive tissue and/or water when compared to smaller/younger animals (**Figures 15 to 17**). This was reported also for *Daphnia* species (Simčič and Brancelj, 1997). Smaller animals are also less tolerant than bigger ones (Brown, 1976; Naylor et al., 1990).

Difference between WW and DW alone represent water, which is taken out from tissues with drying of the whole animal. It still does not tell anything about active tissue content. We wanted to supplement selected methods with a simple method that would help us with active tissue definition. Method of DW determination proved to be inappropriate, considering further use of animal in experiments, since drying makes animal tissue no longer useful for i.e. enzymatic tests. Instead of drying animals and defining DW, different methods that could be used for determination of active tissue were tested. Total phosphorus and total protein content measurement were tested.

Method of total protein content was tested as more suitable as measuring of total phosphorus content like Simčič and Brancelj (2003) suggested. For the measurement of total protein content we could use supernatant residue from ETS method. It proved to be more suitable predictor of metabolically active tissue in individual animals, as we could individually measure with actual value instead of linear approximations from graphs. This protein content was an aid for estimating our choice of animals that were selected for measurements (**Table 16 and 17**). Total protein content value could be used for calculation of ETS activity, but than it would be impossible to compare ETS/R values with results from other authors that used WW (Kenner and Ahmed, 1975a; King and Packard, 1975; Bamstedt 1980; Cammen et al., 1990; Musko et al., 1995; G.-Tóth et al., 1995a and 1995b; Simčič et al., 2005).

4.2.3 Effects of temperature on respiration and ETS/R ratio

ETS and respiration rate are two processes that respond differently on change of temperature. Several researches confirmed correlation between ETS and respiration (King and Packard, 1975; Musko et al., 1995; Simčič and Brancelj, 1997, 2001). Importance of acclimation of test animals is shown with results from ETS/R ratio that is raised with rise in temperature (Bamstedt, 1980; del Giorgio, 1992; Musko et al., 1995). The rise in ETS activity with higher temperature is higher than rise in respiration rate. Bamstedt (1980) observed important influence of temperature to ETS for species *Acartia tonsa*, where animals acclimated at 17 °C had lower ETS activity than animals at 21 °C but both ETS activities were measured at the same temperature. Animals at higher temperature had higher concentration of enzymes and higher ETS values. While for respiration it would mean that after acclimation to lower temperature respiration rate was higher.

ETS activity, which presents metabolic potential, in a process of acclimation to higher temperature, is increasing, so it could ensure higher energetic needs of an organism. After acclimation to higher temperature (i.e. from 10 to 15 °C), respiration is decreased, so energetic losses at respiration would be minimized at the same time.

Respiratory reactions depend also on the environmental temperature at which organisms live. Bamstedt (1980) figured that enzyme concentration inside optimal temperature range is constant, while variation of temperature outside this range leads to unpredictable changes. Organisms act as conformists or regulators; regulators actually actively regulate their metabolic potentials after alteration in temperature outside optimal range. This metabolic potential is dependant on concentration and variability of enzyme sets and is actually a measure of ability of an organism to survive at certain temperature (Packard, 1971). Higher metabolic potential of an organism at certain temperature means precedence against the one with lower metabolic potential. While high increase in respiration rate with raise in temperature means higher energy losses, which is disadvantageous considering growth and reproduction (Goss and Bunting, 1980; Korhonen and Lagerspetz, 1996).

Since the average temperature at our collection sites is around 10 °C we determined it as the most appropriate temperature for all the procedures in our experiments. The same temperature was proposed from Simčič and Brancelj (1997) for *Daphnia* species for measurements with the same methods as we used. Temperature in acclimation chamber was also adapted to experimental conditions. It was tested that tempering pool was able to stay clear for whole weak and animals were able to survive longer in the acclimation chamber at 10 °C. Average annual temperature of water in Slovenia is around 9 to 10 °C, which is important factor for animal acclimation. At the end we found the temperature 10 °C as optimal temperature for microrespirometric measurements.

At the start two populations of same species were tested at 10 °C. Results show that differences between populations of the same species can be smaller than between animals of the same population (del Giorgio, 1992). After that measurements of temperature influence were conducted at two selected temperatures 10 and 15 °C for each selected population of individual species. Both selected test animals had higher ETS/R ratio at 10 °C than later at 15 °C (**Table 10** and **Figure 18**). Musko et al. (1995) showed that rise in temperature means also rise in ETS for *G. fossarum*. In our case of *G. fossarum* ETS/R ratio was raised due to decrease in R and not because of raise in ETS. It means that animals metabolism is already raised at 10 °C and that this temperature could be optimal for measurement of stress caused by exposure to toxicants. Stress caused by selected temperature is not high, since values of ETS/R ratio are still high in average 2.59 ±0.84 at 10 °C and 3.93 ±1.27 at 15 °C. Musko et al. (1995) calculated ETS/R ratios around 1.91 at 10 °C and 1.99 at 15 °C. In the case of *G. fossarum* from the Iščica River, temperature was correctly selected, since ETS values were not raised much and only respiration was affected (**Table 11** and **Figure 18**).

For *A. aquaticus* from Cerkniško jezero ETS/R ratio mean values were higher - from 5.29 ±0.84 at 10 °C and 8.41 ±2.48 at 15 °C. This increase was due to high raise in ETS with temperature raise as suggested by Musko et al. (1995). Also respiration was higher at 15 °C when compared to the one at 10 °C. Adcock (1982) showed similar raise of respiration with raise of temperature from 2 to 18 °C. Edwards and Learner (1960) measured for R 1.5 fold greater values at 20 °C then at 10 °C. We also observed significant raise in respiration. The peak in ETS could mean that animal's optimal temperature is closer to 15 °C. Animal at 10 °C uses and looses more energy for respiration than they would at 15 °C or even higher temperature (Korhonen and Lagerspetz, 1996). Temperature 10 °C was selected for experiments since ETS method in general proved to work better at 10 °C.

Bamstedt (1980) advised that animals collected for ETS activity measurement should not be held in the lab for any length of time. Their ETS activity responds slowly to changes, it stays nearly the same for 3 to 4 days. Since we wanted to measure also oxygen consumption, not only ETS, we had to arrange the whole method to accordance.

Attached bacteria can contribute quite significantly to ETS or R measurement value (Lampert, 1984). So we tried to remove most of attached bacteria and algae from the surface of the animals by rinsing them with distilled water. After first moult animals were visibly cleaner than at the beginning when they were brought from nature. At higher temperatures algal and bacterial growth is also more abundant; quite disturbing can be bacteria inside measuring equipment that can contribute significantly to respiration if surfaces are not cleaned periodically.

4.2.4 Microrespirometer method

Flowing water respirometer with polarographic sensors was used already from year 1953 and they measured also with constant-volume respirometers (Edwards and Learner, 1960). Obviously these methods of measuring oxygen consumption are very old and evolved in many alternatives.

Method of oxygen consumption measurement was changed from microrespirometer with polarographic sensors to new method with optical fiber sensors. The new measurements are much quicker and do not require constant recalibrations like old system did. There is no concern regarding complicated work with polarographic electrodes and the system does not require constant mixing. At Cyclobios microrespirometer electrodes actually consumed oxygen so mixing and flow had to be provided. Actual air pressure is not any more calculated into maximum oxygen concentration in water that is provided to animals, since new system measures actual oxygen value directly at the measuring spot.

The light effect can contribute to stimulation of animals inside the test chamber and higher oxygen consumption as it was proved by experiment when *Niphargus stygius* was exposed to the light (Simčič and Brancelj, pers. com.).

4.2.5 Calorimetry and stress enzymes measuring method

The microcalorimetric method was first used on terrestrial animals like german cockroach (Nielsen et al., 2006) and we tried to develop the method also for aquatic animals. At the start a series of calibration test experiments was performed. Problem with condensation was resolved after putting a container with colder water to the calorimeter. We also had to define animals temperature optimum interval, and after that all experiment were carried out at 15 °C. This temperature was close to room temperature and in optimal range for *G. fossarum* as Pöckl (1993) suggested from the field observations; the range is from 4 to 19 °C. Optimal temperature for microcalorimetric measurements proved to be 15 °C. This was tested with continuous rising of the temperature in DSC microcalorimeter. Animal metabolism at low temperatures is low, with increased temperature it is increased until optimum range is reached. After this optimum range with temperature also metabolism increases up to point of death caused by heat shock. With a process called acclimation animal can change this temperature range until a certain genetically defined point (Korhonen and Lagerspetz, 1996).

Glutathione S-transferase (GST) and glutathione peroxidase (GPOx) activities were tested. Most of arthropods contain multiple forms of GST and GPOx enzymes with different substrate specifities (Lamoureux and Rusness, 1989). Most experiments regarding co-substrate glutathione have been conducted on mammals and target insects for certain pesticide. It is interesting that these enzymes are both inducible like reported for terrestrial wolf spider (Nielsen et al., 1999). Enzyme production can be induced quickly after exposure. Both methods used for testing stress enzyme activity were evolved in many varieties considering use of different substrates. We used chloro-di-nitro-benzene (CDNB) as a substrate for glutathione S-transferase and H_2O_2 for glutathione peroxidase. Most authors used substrates for assay of the various GST isoenzymes from arthropods CDNB and/or di-chloro-nitro-benzene (DCNB) with the glutathione co-substrate (Lamoureux and Rusness, 1989). For GPOx the main substrate was H_2O_2 with glutathione co-substrate. Glutathione conjugation with use of those substrates in both methods was used as a predictor of stress enzyme activity.

4.3 Exposure to toxic substances

4.3.1 Toxic substances and stress

The peak concentrations of chemicals are usually present for a short time in the water bodies, especially running ones, and they usually do not kill populations of biota present there. However, their influence can be disturbing in many ways for most of animals and plants present (de Oliveira-Filho et al., 2004). It is proposed that utilization of invertebrates as target organisms in water quality assessment could bring more to health of aquatic ecosystem than, for example data acquired only from fish tests. Therefore it is necessary to develop new methods with different test animals for more integral view of short pulse effects (Cairns, 1983). It is important to measure changes at conditions as close as possible to those present in the nature. Short pulses of extremely high concentrations can be observed for a short period, after that concentration is diluted in larger water body. Use of acute concentrations higher than LC_{50} for shorter period is advised (Cold and Forbes, 2004).

Many authors have worked on determination of lethal exposure concentration of several animal species; most of them performed simple LC_{50} tests. We used the same base and further expanded this method with various stress determination methods.

Different species of test organisms are not equally susceptible to the same toxic substance nor are they equally susceptible in different stages of their life cycle. Important matter is also previous exposure to toxicants; it may also change susceptibility of organisms and cause their adaptations. Organisms of the same species can respond differently to the same level of a toxicant from time to time, even when other test conditions are kept the same (Selye, 1975; Brown, 1976; Clesceri et al*.,* 1998).

4.3.2 Toxicity tests

Effects of chemical interactions are complex and toxicity can not be determined from chemical tests alone. Acute toxicity tests were done for all four allochthonous substances for range finding and accurate definition of LC_{50}. We measured physiological reactions after exposures in nominal concentrations that were renewed periodically. The concentration did not drop so much from the starting value. The concentration of the toxicants in the nature is reduced in the same way as in test solutions in the laboratory with similar decomposition route and half life. For further experiments we would advice additional analytical analyses for actual substance content in exposure solutions and in animal tissue. They would give additional information on fate of selected chemicals in animal's bodies.

For each substance minimal legislation data were added in order to show what concentration is allowed in aquatic ecosystems. We compared our results from toxicity tests with results from other authors. Comparison is always a bit partial, since experimental conditions are usually not the same, but still they can tell something about concentration that we used in our experiments. Measurements of effects on ETS and R after exposure to toxic substances were compared to those few researches from other authors that had been accomplished on ecotoxicological field. Our results compared with reports from other authors together with LC_{50} are discussed at each individual toxic substance (from 4.3.3 to 4.3.6).

4.3.3 Copper sulfate

Copper sulfate is water soluble and it doesn't have additional solvent or metabolite that can be toxic to animals. It was interesting starting substance for studying influences of different substances on respiration processes. We speculated that since copper influences mitochondrial membranes (site of electron transport system) it would also affect ETS activity.

US EPA defined maximum contaminant level for copper at 1.3 mg l^{-1} of copper in water quality assessment for human safety reason (US EPA, Federal register). Our national standard is 2 mg l^{-1} and it is normally not exceeded. Quality classes for surface water (in a table scored from 1 to 4) define concentrations for grade 1: less than 30 µg l^{-1}, grade 2: 30 to 100 µg l^{-1} and grade 3: 100 to 140 µg l^{-1},

while concentrations higher than 140 µg l^{-1} define water quality group 4 (MOP-HMZ, 2000). Concentrations used in our tests were higher and can still be reached just at catastrophic events, when storm water brings loads of substance to aquatic system.

Chronic exposure of *A. aquaticus* juveniles and females was tested at concentration 5 µg l^{-1} for mortality and growth reduction (De Nicola Giudici et al., 1988). Concentration of 12.1 µg l^{-1} of copper after exposure for 96h was shown effective in reduction of feeding at juveniles of *G. pulex* (Blockwell et al., 1998). From our results we could conclude that chronic exposure of copper sulfate in range of grade 1 in aquatic system already has influence on water biota.

Acute toxicity tests were performed by De Nicola Giudici et al. (1987) LC_{50} was investigated for 36 days with concentrations ranging from 0.01 up to 15 mg l^{-1} of copper on *A. aquaticus.* Adult males showed LC_{50} at high concentration 0.01 mg l^{-1} (30 days), at higher concentration of copper (up to 1 mg l^{-1}) LC_{50} effects were reduced to 23 - 18 days and at concentration 5 mg l^{-1} of copper it fell to 6 days. Females LC_{50} were parallel to male's ranging from 35 days to 6 days at treatments higher than 5 mg l^{-1} of copper. Brown (1976) used from 0.1 up to 5 mg l^{-1} of copper and determined LC_{50} (48h) from 1.2 up to 2.5 mg l^{-1} of copper for different populations of *A. meridianus.* Migliore and De Nicola Giudici (1990) reported sulblethal concentration between 2 and 11 mg l^{-1} as LC_{50} for *A. aquaticus* at between 8 and 18 days. Mulliss et al. (1994) reported increased mortality in regard to copper. Copper is the most important heavy metal toxicant with respect to mortality of *A. aquaticus* in urban streams. The most probable way of intake is considered ingestion of algae, which can assimilate copper and also organic complexes (biomagnification). Mulliss et al. (1996) confirmed his results again with measurements of chronic exposure. Mean ratio of copper bioaccumulation after 36 days in *A. aquaticus* was 1.95 and in *G. fossarum* 2.37 fold the value at the start of experiment. Measured value of dissolved aqueous copper in the experiment was in wet weather days 39.4 mg l^{-1} (total copper was 88.9 mg l^{-1}) and in dry weather days 35.2 mg l^{-1} (total copper 59.9 mg l^{-1}). Martin and Holdich (1986) defined LC_{50} (96h) at concentration 9.2 mg l^{-1} (5.6 to 36.4 mg l^{-1}) for *A. aquaticus*, while Furmanska (1979) determined LC_{50} (48h) at concentration of 32.1 mg l^{-1} of copper.

Our results from *A. aquaticus* showed LC_{50} (48h) values at concentration 30 mg l^{-1}, which is in accordance with Furmanska (1979), while EC_{50} (24h) concentration was 20 mg l^{-1} of copper.

Bamstedt (1980) reported small but significant increase in ETS at concentration 10 µg l^{-1} of copper at mixed zooplankton acclimated for 40h to experimental conditions. In our experiments the effect of exposure to copper in on R and also ETS was insignificant. In a case, that both measured values are decreased due to exposure copper ions, the calculated ETS/R ratio would stay nearly the same. In this way we actually get false negative result after exposure if just ETS/R parameter is taken on the end for estimation of the stress. In our case ETS activity was higher at group 3 (5 mg l^{-1} of copper) and again lower at group 4 (10 mg l^{-1}), while at the same time R was the same (**Table 13**). Results show also a small insignificant reduction in ETS/R (**Figure 19**).

4.3.4 Imidacloprid

EPA regulation standard for imidacloprid was set for surface water at 35.89 µg l^{-1} and for groundwater 17.24 µg l^{-1}. Standard for ground water is lower, since ground water can be directly provided to consumers without treatment. Assessment showed potential risk at estimated acute exposure concentration in surface water at 36.04 µg l^{-1} and for groundwater at 2.09 µg l^{-1} of imidacloprid. For chronic exposure value for surface water is lower; at 17.24 µg l^{-1} of imidacloprid defined on dietary exposure of human population (US EPA, Federal register). These are US legislation standards; our country has limited value of imidacloprid to 0.1 µg l^{-1} and daily measured values are c. 0.05 µg l^{-1}. Since imidacloprid is still available on Slovenian market, right after application on fields with the drain of storm-water, concentrations used in our experiments for a short period could be reached.

Song et al. (1997) reported that imidacloprid can be used with environmental safety regarding freshwater arthropods. They used only toxicity tests for determination of LC_{50} and did not follow any of physiological indicators of stress.

One hour exposure procedure was introduced with this pesticide due to fresh set of data acquired from references (Cold and Forbes, 2004). Animals during our experiments were exposed for one hour.

Song et al. (1997) determined for *Daphnia magna*, that at 27 °C within acute toxicity tests concentration of LC_{50} (48h) is 10.4 mg l^{-1} and for *Artemia* sp. 361.2 mg l^{-1}. At lower temperature (at 20 °C) these values were defined only for *Daphnia magna* and are higher; LC_{50} (48h) concentration is 17.4 mg l^{-1}. Sánchez-Bayo and Goka (2006) determined LC_{50} (48h) from 65 up to 133 mg l^{-1} for *Daphnia* sp.. Bacey (2002) reported LC_{50} (48h) values for *Daphnia magna* as 85 mg l^{-1} and Mysid shrimp LC_{50} (96h) as 34 mg l^{-1}. That is much higher compared to our LC_{50} concentration, determined at 10 mg l^{-1} of imidacloprid for *A. aquaticus* and 1 mg l^{-1} for *G. fossarum*. Our LC_{50} values are also lower compared to Song et al. (1997). Jemec et al. (2007) in their comparative survey proved that imidacloprid alone is less toxic to *D. magna* than commercial liquid formulation Confidor SL 200 that was used also in our case. They also showed that different aquatic arthropods can be even more susceptible to imidacloprid compared to *D. magna.* They also observed similar LC (as LOLC – lowest observed lethal concentration) at 10 mg l^{-1} of imidacloprid for Confidor SL 200, which is the same as LC_{50} for our less susceptible species *A. aquaticus* (**Table 12**).

Effective concentrations that we got for *A. aquaticus* was 1 mg l^{-1} and for *G. fossarum* 0.1 mg l^{-1} of imidacloprid; latter ones are in accordance with Kreutzweiser et al. (2007) that reported high mortality of aquatic insects at concentration of 0.13 mg l^{-1} and significant feeding inhibition at 0.012 mg l^{-1} for imidacloprid applied directly to aquatic microcosms. They measured this concentration after it was well distributed in water system, and did not include an effect of starting high peak concentration on aquatic insects.

Calculated ETS/R ratio shows a significant effect on *A. aquaticus* after one hour exposure at group 4 (1 mg l^{-1}) and group 5 (10 mg l^{-1} of imidacloprid) (**Figure 20**). Respiration was significantly increased in both groups, in group 5 ETS was also significantly lower, which means that imidacloprid in higher concentrations influences ETS activity, too.

For *G. fossarum* ETS/R ratio was significantly lower in two exposed groups (0.01 mg l^{-1} and 10 mg l^{-1} of imidacloprid), which could mean that it is even more susceptible than *A. aquaticus*. Since the difference was also not significant in 1 mg l^{-1} group, it is not absolutely proved (**Figure 20**). Respiration values were equivalent and it was not raised in any group, but ETS was significantly lower in group 4 (10 mg l^{-1}) and also a bit lower in other two exposed groups. Groups average WW show that control group 1 and group 4 have similar WW and can be compared due to similar active tissue content, while group 2 and 3 have a bit higher average WW and this could contribute to higher ETS activity (**Table 15**).

Our results show that already sublethal concentrations in short pulse exposures can cause effects (**Table 14 and 15**). The effect was not seen at raised respiration rate, but in fall of ETS activity at both test animals. That indicates that imidacloprid at higher concentrations in quite short exposure period, actually affects enzymatic level in cells and not oxygen consumption. Imidacloprid at concentrations higher than 10 mg l^{-1} influenced rise in respiration as well as decrease in ETS activity at both tested animal species.

G. fossarum is more sensitive species compared to *A. aquaticus* (Hargeby, 1990; Graça et al., 1994b). In our experiments *G. fossarum* proved to be more sensitive to imidacloprid exposure when compared to *A. aquaticus*. This was proved in 48 hour exposure toxicity test as well as in respiration measurements after short one hour exposure.

Jemec et al. (2007) suggested use of broader spectrum of aquatic test animals before imidacloprid will be classified as a safer than currently appied pesticide. Our contribution is in compliance of this suggestion.

4.3.5 Atrazine

EPA regulation standard for atrazine was set for freshwater as one hour average concentration at 0.350 mg l^{-1} (acute exposure). For chronic exposure value was defined as four day average concentration in water at 0.012 mg l^{-1} of atrazine defined on dietary exposure of human population (US EPA, Federal register). Officially measured runoff waters from treated cornfield were reported that they can exceed 0.74 mg l^{-1}. A proposed criterion from year 1989 for most aquatic plants and animals is <11 µg l^{-1} (Eisler, 1989). This criterion was set in days when atrazine was widely used all around the world. It is still one of the most widely used agricultural pesticides in United States. There is a reregistration process pending in several countries arround the world. It is proposed that some farmers can still have it and they could still be using it, regardless the ban of sell in Slovenia.

Official standard in Slovenia is $1x\ 10^{-4}$ mg l^{-1} and for all pesticides combined $5x\ 10^{-4}$ mg l^{-1} in drinking water (MOP - HMZ, 2000). Atrazine use was banned in Slovenia from year 1996, but residues can still be found in surface and ground waters. For this reason high acute concentrations that were used in our experiments can not be reached in nature not even for a short period of time any more.

Munn and Gilliom (2001) defined atrazine effective concentrations EC_{50} (24h) for freshwater invertebrates. Effective concentrations for *Gammarus pulex, G. italicus, Daphnia pulex* and *Hyalea azteca* were reported 14.9, 10.1, 41.5, 14.7 mg, respectively. We defined EC_{50} (24h) for both test species at concentration 0.1 mg l^{-1}. Those 24h concentration are about 100-times higher then ours. We could conclude that our test animals were more susceptible to atrazine.

Pantani et al. (1997) defined LC_{50} (96h) of 10.1 mg l^{-1} of atrazine for *Gammarus italicus* and for *Echinogammaus tibaldii* at LC_{50} (96h) of 3.3 mg l^{-1} of atrazine. Our LC_{50} (48h) was defined at 30 mg l^{-1} of atrazine for *A. aquaticus* and 10 mg l^{-1} of atrazine for *G. fossarum*. It is difficult to compare LC_{50} (96h) and LC_{50} (48h), but if we compare *G. fossarum* LC_{50} (48h) value it is already the same as LC_{50} (96h) from *G. italicus.* That means that *G. fossarum* is more susceptible to atrazine than *G. italicus* and *A. aquaticus*.

Acute one hour exposure to atrazine at the concentration of 10 mg l^{-1} (group 3) for *A. aquaticus* showed influence on R and no effect on ETS for this reason ETS/R ratio is also significantly lower (**Table 16 and Figure 21**). There was no observed effect at lower exposure concentration (5 mg l^{-1}).

For *G. fossarum* ETS/R ratio for all exposed groups is significantly lower due to higher R. R was significantly increased in group 3 (3 mg l^{-1}) and group 4 (10 mg l^{-1}). ETS was consistent also if compared to all other experiments. WW of all groups was similar and did not differ significantly. Total protein content was indicator of homogeneity among groups (**Table 17**). All treated groups had significantly lower ETS/R ratio compared to control. Exposures of *G. fossarum* at concentrations higher than 1 mg l^{-1} of atrazine show a significant effect (**Figure 21**).

G. fossarum is more susceptible to atrazine than *A. aquaticus*. This is shown with toxicity test as well as with respiration measurements. Toxicity tests have indicated that 48 hour exposures to 10 mg l^{-1} of atrazine have lethal effect on *G. fossarum* (LC_{50}) and not on *A. aquaticus*, while short one hour exposure to 10 mg l^{-1} of atrazine shows an effect on respiration of both tested animals. Difference between both tested species was shown at lower concentrations. *G. fossarum* was shown to be sensitive also to lower concentrations (1 and 3 mg l^{-1}) of atrazine.

4.3.6 Esfenvalerate

Acute drinking water exposure is estimated for the U.S. population to be 0.039 µg/kg/day. For non-nursing infants less than 1 year old the exposure is 0. 074 µg/kg/day. The chronic drinking water exposure is estimated to be of maximum value of 0.001 µg/kg/day and for the non-nursing infants 0.005 µg/kg/day (US EPA, Federal register).

High concentrations have already been found in Danish water bodies in the range of 0.03-0.2 µg/l, which is high enough to reduce the abundance of many insect species (Pan-Europe, 2000). So far in Slovenia we don't even have legislations and testing for pesticide esfenvalerate in waters. Exceptions are those for pesticide residues in food.

Esfenvalerate is highly toxic to freshwater animals like fish (in concentrations from 0.3 to 1 µg l^{-1}) and *Daphnia magna* (1 µg l^{-1}) (Aarhus Amt. 1999, cit. by Forbes and Cold, 2005).

Esfenvalerate exposure was done on *G. fossarum* only. In previous years in Roskilde lab several experiments were done with local species *G. pulex*. Mostly stress enzyme activity measurements were done after exposure to esfenvalerate. A comparison of results between *G. fossarum* brought from Slovenia and local *G. pulex* species was the main idea.

Fairchild et al., (1992) reported decrease of macroinvertebrates at concentrations 0.25 µg l^{-1} of esfenvalerate in field aquatic mesocosms. Cold and Forbs (2004) defined LC_{50} (48h) at 0.142 µg l^{-1} for large individuals (10-14mm) and 0.137 µg l^{-1} for small animals (7-8mm) of *Gammarus pulex*. Our measured LC_{50} (48h) was 0.3 µg l^{-1} for *G. fossarum.* The value is twice higher than reported from *G. pulex,* which could mean that *G. fossarum* is less susceptible to stress caused by esfenvalerate than *G. pulex*. Lozano et al. (1992) reported drastic reductions or elimination of most crustaceans and chironomids after exposures of 1 to 5 µg l^{-1} of esfenvalerate.

Selected *G. fossarum* individuals for measurements in microrespirometer were smaller as their WW ranged from 8 to 9 mg. Smaller animals were desired, since the container with animal is small (1 cm^3) and also amount of water that can be provided into container is max. 750 µl. When compared to other experiments (i.e. *G. fossarum* exposed to atrazine), when WW ranged from 14 to 16 mg. Although animals were smaller they were all adult males, WW between the groups did not differ significantly. Total protein content also indicates homogeneity among tested groups.

Calorimetric measurements
Animals that are exposed to toxic substance like esfenvalerate for one hour exhibit elevated heat production. Their metabolism adapts to stress conditions with raised respiration and production that helps animal at detoxification process and to survive. Differences after exposure measured at the same animals show significant raise of heat production with raise of exposure concentrations. There is significant difference in all groups with concentration higher than 0.01 µg l^{-1}. The same result was gained from data expressed with total protein content as with calculations of DW derived from WW. One hour exposure concentrations higher than 0.1 µg l^{-1} show an effect on test animals through elevated heat production (**Table 18, Figure 22**).

Stress enzymes
G. fossarum GST activity (**Table 18, Figure 23**) shows no significant raise in all groups, although if we actual figures and not logarithmic values are compared. There is a raise of GST activity in group 5 at concentration of 0.3 µg l^{-1} of esfenvalerate. That means that probably at exposure concentrations higher than 0.3 µg l^{-1} GST activity would also be elevated after longer exposure period. Lamoureux and Rusness (1989) pointed out that different insect species and strains vary greatly in their ability to metabolize and detoxify different insecticides by glutathione conjugation. This could be true also for non-target animal species. Insecticide resistant and susceptible strains can be observed between insects. Among non-target species elevated levels of GST activity could be indication of adaptation to toxic substance.

GST activity in this test was from 3400 up to 4800 nmol min^{-1} mg^{-1} protein. It is not possible to compare this specific enzyme activity to other authors that used other test animals (mostly terrestrial insects) and other methods with different substrates. Only results from experiments following the same protocol and related organisms can be compared. Nielsen et al. (2006) observed GST activity from cockroaches from 4 to 11 nmol min^{-1} mg^{-1} of protein and Nielsen at al. (1999) measured activity from wolf spider ranging from 100 to 300 nmol min^{-1} mg^{-1} of protein. Our values were higher (3400-4800 nmol min^{-1} mg^{-1} protein), which could indicate that freshwater invertebrates have higher basic level of GST activity. Stenersen et al. (1987) indicated quite the opposite with experiments on nine phyla of terrestrial and aquatic animals.

GPOx exhibited significant raise (**Table 18, Figure 24**) of activity at exposure concentrations higher than 0.1 µg l^{-1}. All groups showed raise in enzymatic activity when animals were exposed to esfenvalerate. Measured GPOx activity ranging from 15 to 54 $nmol^{-1}$ min^{-1} mg of protein is in accordance Nielsen at al. (1999) that was from 20 up to 150 nmol min^{-1} mg^{-1} of protein at wolf spider exposed to pesticide cypermethrin.

This experiment indicates that enzyme system activity is induced after one hour exposure to esfenvalerate. Already after one hour there is a raise in GPOx activity, which is probably an answer to induced oxygen stress with oxygen reactive species or to detoxification process involving metabolism of xenobiotics. If stress continues also other systems are activated in animal tissues. It would be energetically uneconomical to activate all systems at once, especially if animal is in bad environment.

Measurement of stress enzyme activity combined with direct calorimetry is a good method in ecology and ecotoxicology for estimation of metabolism rates. Direct calorimetry in combination with measurement of oxygen consumption or CO_2 production would be a good idea for further experiments. The idea is for instance to put flow through direct calorimeter that would measure heat changes inside the test chamber in combination with oxygen meter that would measure oxygen before and after the chamber. Both systems would bring more individual information and would enable test animals to spend more time in the test chamber. Metabolic products would be washed out of the test chamber; the same as in the case of microrespirometer that we used.

4.4 Conclusions

Testing pesticides and other toxic substances and determining real concentrations in nature is difficult, especially in running waters. High toxic peaks can easily be overlooked because of fast dilution through water body. That is why acquiring of additional methods for determination of stress state on selected test animals is necessary.

All the methods used in first and in second part of the present work are good predictors of stress caused by allochthonous substances. Combining them and making standards on a population of test species could be a good contribution to better knowledge on consequences of pollutants in ecotoxicological research.

Combination of R and ETS is a good predictor of stress that can be used on any selected species brought from nature. Stress is shown in reduction of ETR/R ratio at measured animals after exposure to toxic substances. ETS/R ratio values at animals in stress are closing 1 (from higher values up to 10), which demonstrates that they are using 100% of respiratory potential.

Combination of microcalorimetry coupled with stress enzyme measurement is also a good predictor of stress. The state of individual animal can be assessed after exposure to toxic substance. Differences in calorimetric values and stress enzyme activity at exposed animals are higher compared to control group.

High one hour exposure concentrations are in compliance with high peaks in natural systems receiving i.e. stormwater from fields that have been sprayed by selected pesticide.

A. aquaticus and *G. fossarum* are both susceptible to imidacloprid exposure at concentrations higher than 1 mg l^{-1}. *G. fossarum* is affected already at 0.1 mg l^{-1}, which is observed with movement reduction. Furthermore a raise in R and diminution of ETS activity consequently results in lower ETS/R ratio, which is a measure of stress. *G. fossarum* is shown to be more susceptible to imidacloprid exposure than *A. aquaticus.*

Both test animals *A. aquaticus* and *G. fossarum* are also susceptible to one hour atrazine exposure; *A. aquaticus* at concentrations higher than 5 mg l^{-1} and *G. fossarum* at concentrations higher than 1 mg l^{-1}. Both test species demonstrated higher R values and constant ETS values after exposure and consequently lower ETS/R ratio at higher exposure concentrations. *G. fossarum* is shown as more susceptible species to atrazine exposure when compared to *A. aquaticus*.

For the esfenvalerate the effect on *G. fossarum* of concentrations greater than 0.1 µg l^{-1} are significant. This pesticide has adverse effects already in extremely low exposure concentrations. Together with rise of exposure concentrations also raise in calorimetric values and glutathione peroxidase activity was observed. Metabolic rate in animals was raised due to raised respiration and production of detoxifying enzymes and metabolites.

As a recommendation for further experiments we would like to compare *G. fossarum* to related species *G. pulex* and also to *A. aquaticus* as in our former experiments. It would be also interesting to check out stress enzyme activity for more freshwater test animals, which would show if aquatic animals have raised level of stress enzymes activity compared to terrestrial arthropods.

Some additional interesting methods could be joined in with selected methods; one of them is measurement of pleopod beats. This simple method can contribute to visible evaluation of stress to test animals. Reported values from gammarid species from Dutch waters (*G. fossarum, G. pulex, G. roeseli, G. tigrinus, Echinogammarus ischnus* and *Dikerogammarus villosus*) were from 0 beats per minute at 1 °C to maximum of up to 300 beats per minute at temperatures between 25 and 35 °C (Winjhoven et al., 2003; Maltby, 1995). Our observations were in compliance with this. So for our future experiments we could include this method with ETS activity and R rate measurement for confirmation of acquired R results.

Finally the methods presented in this work should be practically implicated. Primarily through bringing loaded water from nature and testing it with test animals from laboratory against the control. In secondary phase it can be tested on animals brought from fresh waters in nature (from polluted and non-polluted parts) and compare them against laboratory animals of the same species. In this way our methods could be suitable for monitoring of environmental pollution.

5. REFERENCES

Adcock J.A. 1982. Energetics of a population of Asellus aquaticus (Crustacea, Isopoda): respiration and energy budgets. Freshwater biology 12 (3):257-269

Ahmad S., Beilstein M.A., Pardini R.S. 1989. Glutathione peroxidase activity in insects: A reassessment. Archives of insect Biochemeistry and Physiology 12: 31-49

Bacey J. 2000. Environmental fate of Imidacloprid. Environmental Monitoring & Pest Management Branch; Department of Pesticide Regulation, Sacramento, Ca 95814 i.p. address: http://www.cdpr.ca.gov/docs/emon/pubs/fatememo/imid.pdf (22.01.2008)

Bamstedt U. 1980. ETS activity as an estimator of respiratory rate of zooplankton populations. The significance of variations in environmental factors. Journal of Experimental Marine Biology and Ecology 42: 267-283

Berg J.M., Tymoczko J.L., Stryer L. 2002. Biochemistry: II Transducing and storing energy, WH Freeman and Co., New York. Chapter 19; The light reactions of photosynthesis p. 2704-2716

Boulanger B., Nikolaidis P. 2003. Mobility and aquatic toxicity of copper in an urban watershed. Journal of the American Water Resources Association 39 (2): 325-336

Borgmann U. 1977. Electron transport system activity in Daphnia and crayfish Canadian journal of zoology 55: 847-854

Blockwell S.J., Taylor E.J., Jones I., Pascoe D. 1998. The influence of fresh water pollutants and interaction with *Asellus aquaticus* (L.) on the feeding activity of *Gammarus pulex* (L.). Archivum of environmental contamination and toxicology 34 (1): 41-7

Brown B.E. 1976. Observations on the tolerance of the isopod *Asellus meridianus* Rac. to copper and lead. Water research 10: 555–559

Brown B.E. 1977. Uptake of copper and lead by a metal-tolerant isopod *Asellus meridianus* Rac. Freshwater Biology 7: 235 244

Brusca R.C., Brusca G.J. 2003. Phylum Arthropoda: The Crustacea. Second edition, Sinauer Associates, Inc., Sunderland, Massachusetts; p. 511-587

Cairrão E., Couderchet M., Soares A.M.V.M., Guilhermino L. 2004. Glutathione-S-transferase activity of *Fucus* spp. As a biomarker of environmental contamination. Aquatic Toxicology 70: 277-286

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

Cairns J. Jr, Niederlehner B.R. 1987. Problems associated with selecting the most sensitive species for toxicity testing Hydrobiologia 153 (1): 87-94

Cammen L.M., Corvin S., Calow P. 1990. Electron transport system (ETS) activity as a measure of benthic macrofaunal metabolism. Marine ecology progress series 65(2): 171-182

Campbell N.A., Rees J.B., Mitchell L.G. 1999. Biology. Fifth edition Glenview, Illinois

Cezilly F., Gregoire A., Bertin A. 2000**.** Conflict between co-occurring manipulative parasites? An experimental study of the joint influence of two acanthocephalan parasites on the behavior of *Gammarus pulex.* Parasitology 120: 625-630 Cambridge University Press

Christensen J.P., Packard T.T. 1979. Respiratory Electron Transport Activities in Phytoplankton and Bacteria: Comparison of Methods Limnology and oceanography 24 (3): 576-583

Chapman P., Mitchell-Innes B.A., Walker D.R. 1994. Microplankton ETS measurements as a means of assessing respiration in the Benguela ecosystem. South African journal of marine science 14: 297-312

Chiron S., Fernandez-Alba A.R., Rodriguez A., Garcia-Calvo E. 2000. Pesticide chemical oxidation: State-of-the-art. Water research 34 (2): 366-377

Clesceri L.S., Greenberg A.E., Eaton A.D. 1998. Standard methods for the examination of water and wastewater, Chapter Toxicity 8000 and Chapter 4500-O Twentieth edition APHA, AWWA, WEF

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

del Giorgio P.A. 1992. The relationship between ETS (electron transport system) activity and oxygen consumption in lake plankton: a cross-system calibration. Journal of Plankton research 14 (12): 1723-1741

De Nicola Giudici M., Migliore L., Gambardelle C., Marotta A. 1988. Effect of chronic exposure to cadmium and copper on *Asellus aquaticus* (L.) (Crustacea, Isopoda). Hydrobiologia 157: 265-269

De Nicola Giudici M., Migliore L., Guarino S.M. 1987. Sensitivity of *Asellus aquaticus* (L.) and. *Proasellus coxalis* Dollf. (Crustacea, Isopoda) to copper. Hydrobiologia 146: 63-63

de Oliveira-Filho E.C., Lopes R.M., Paumgartten F.J.R. 2004. Comparative study on the susceptibility of freshwater species to copper-based pesticides. Chemosphere 56 (4): 369-374

Dorit R.L., Walker W.F., Barnes R.D. 1991. Zoology, Saunders College Publ., Philadelphia, USA.

Edwards R.W., Learner M.A. 1960. Some Factors Affecting the Oxygen Consumption of *Asellus.* Journal of Experimental Biology 37:706-718

E.I. duPont de Neumors and Co, 2002. DuPont Asana XL Insecticide: Technical bulletin. Review report for the active substance esfenvalerate. European commission: Directorate-General health and consumer protection.

Eisler R. 1989. Atrazine hazards to fish, wildlife and invertebrates: A synoptic review. Contaminant hazard reviews biological report 85 (18): 1-34

EPA US GOV 2003 i.p.adress: Atrazine Interim reregistration eligibility decision (IRED) http://www.epa.gov/oppsrrd1/reregistration/atrazine (03.03.2008)

EPA US GOV i.p.adress: atrazine http://www.thecre.com/atrazine/fedactions.htm#sdwastandards
http://www.epa.gov/oppsrrd1/reregistration/atrazine
copper http://www.epa.gov/EPA-PEST/2000/November/Day-15/p28715.htm
esfenvalerate http://www.epa.gov/EPA-PEST/1998/September/Day-11/p24770.htm
imidacloprid http://www.epa.gov/EPA-PEST/2003/June/Day-13/p14880.htm
(01.03.2008)

Fairchild J.F., La Point T.W, Zajicek J.L., Nelson M.K., Dwyer F.J., Lovely P.A. 1992. Population-, community- and ecosystem-level responses of aquatic mesocosms to pulsed doses of a pyrethroid insecticide. Environmental Toxicology and Chemistry 11: 115-129

Falkner K.C., Clark A.G. 1992. The effects of temperature and pH on the reaction between 1-chloro-2,4-dinitro-benzene and glutathione, catalyzed by the major glutathione S-transferase from *Galleria melleonella*. Insect Biochemistry and Molecular Biology 22 (8): 917-923

Fenton G.W., Summers C.B. 1995. Antioxidant Systems in Insects. Archives of Insect Biochemistry and Physiology 29: 187-197

Ferrari MJ, Ator SW, Blomquist JD, Dysart JE, 1997. Pesticides in surface water of the MID-Atlantic region. U.S. geological survey, box 25286, Denver federal centre, Denver

Fernandez-Alba A.R., Guil M.D.H., Lopez G.D., Chisti Y. 2002. Comparative evaluation of the effects of pesticides in acute toxicity luminescence bioassays Aanalitica chimica acta 451 (2): 195-202

Forbes V.E., Cold A. 2005. Effects of the pyrethroid esfenvalerate on life-cycle traits and population dynamics of *Chironomus riprus* – importance of exposure scenario. Environmental Toxicology and Chemistry 24 (1): 78-86

Furmanska M. 1979. Studies of the Effect of Copper, Zinc and Iron on the Biotic Components of Aquatic Ecosystems. Polskie Archiwum Hydrobiologii 26(1/2): 213-220

Gnaiger E., Forstner H. 1983. Polarographic Oxygen Sensors, Aquatic and Physiological Applications. Springer-Verlag Berlin Heidelberg New E, Forstner H, 1983. Polarographic Oxygen Sensors, Aquatic and Physiological Applications. Springer-Verlag Berlin Heidelberg

Goss L.B., Bunting D.L 1980. Temperature effects on zooplankton respiration. Comparative Biochemistry and Physiology 66A: 651-658

Gould F. 1991. The evolutionary potential of crop pests. American science 79: 496-507

Graça M.A.S., Maltby L., Calow P. 1994a. Comparative ecology of *Gammarus pulex* (L.) and *Asellus aquaticus* (L.) I: population dynamics and microdistribution. Hydrobiologia 281 (3): 155-162

Graça M.A.S., Maltby L., Calow P. 1994b. Comparative ecology of *Gammarus pulex* (L.) and *Asellus aquaticus* (L.) II: fungal preferences. Hydrobiologia 281 (3): 163-170

Graebing P., Chip J.S. 2004. Soil Photolysis in a Moisture- and Temperature-Controlled environment. 2. Insecticides. Journal of Agricultural and Food Chemistry 52: 2606-2614

G.-Tóth L. 1993. Electron transport system (ETS) activity of the plankton, sediment and biofilm in Lake Balaton (Hungary) Verhandlung international verein of limnology 25: 680-681

G.-Tóth L., Carrillo P., Cruz-Pizarro L. 1995a. Respiratory electron transport system (ETS)-activity of the plankton and biofilm in the high-mountain lake La Caldera (Sierra Nevada, Spain). Archiv fur Hydrobiologie 135 (1): 65-78

G.-Tóth L., Szabo M., Webb D.J. 1995b. Adaptation on the tetrazolium reduction test for the measurement of the electron transport system (ETS) activity during embryonic development of medaka. Journal of fish biology 46: 835-844

Guzsvany V., Csanadi J., Gaal F. 2006. NMR study of the influence of pH on the persistence of some neonicotinoids in water. Acta chimica slovenica 53: 52-57

Habig W.H., Pabst M.J., Jakoby W.B. 1974. Glutathione S-Transferases. The Journal of Biological Chemistry 249 (22): 7130-7139

Hargeby A. 1990. Effects of pH, humic substances and animal interactions on survival and physiological status of *Asellus aquaticus* L. and *Gammarus pulex* (L.). A field experiment Oecologia 82 (3): 348-354

Hart Scientific 1991. Differential Scanning Calorimeter, User manual. Pleasant groove, Utah

Hartley D., Kidd H. 1983. The agrochemicals handbook. Nottingham, England: Royal Society of Chemistry

Huckins J.N., Petty J.D., England D.C. 1986. Distribution and impact of trifluralin, atrazine and fonofos residues in microcosms simulating a northern prairie wetland. Chemosphere 15 (5): 563-588

Hynes H.B.N. 1954. The ecology of *Gammarus duebeni* Lilljeborg and its occurrence in freshwater in western Britain. Journal of Animal Ecology 23: 38–84.

ISO-standard 6341:1996 (E). Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea)-Acute toxicity test - Third edition International Organization for Standardization, Geneve.

Jemec A., Tišler T., Drobne D., Sepčić K., Fournier D., Trebše P. 2007. Comparative toxicity of imidacloprid, of its commercial liquid formulation and diazinon to non-target arthropod, the microcrustcean *Daphnia magna.* Chemosphere 68: 1408-1418

Karaman G.S., Pinkster S. 1977. Freshwater *Gammarus* species from Europe, North Africa and adjacent regions of Asia (Crustacea-Amphipoda) Part I. *Gammarus pulex*-group and related species

Kenner R.A., Ahmed S.I. 1975a. Measurements of electron transport activities in marine phytoplankton. Marine biology 33 (2): 119-127

Kenner R.A., Ahmed S.I. 1975b. Correlation between oxygen utilization and electron transport activity in marine phytoplankton. Marine biology 33: 129-133

King F.D., Packard T.T. 1975. Respiration and respiratory electron system in marine zooplankton. Limnology and Oceanography 20: 846-854

Korhonen I.A., Lagerspetz K.Y.H. 1996. Heat shock response and thermal acclimation in *Asellus aquaticus*. Journal of Thermal Biology 21 (1): 49-56

Kreutzweiser D., Good K., Chartrand D., Scarr T., Thompson D. 2007. Non-target effects on aquatic organisms of imidacloprid as a systemic insecticide to control emerald ash borer in riparian trees. Ecotoxicology and Environmental Safety 68 (3): 315-325

Krohn J., Hellpointner E. 2002. Environmental fate of imidacloprid. Pflanzenschutz nachrichten Bayer 55 (special edition): 1-26

Lampert W. 1984. The measurement of respiration. In: Downing JA, Rigler FH, editors, A Manual on Methods for the Assessment of Secondary Productivity in Fresh Waters. IBP Handbook no. 17, Oxford: Blackwell Scientific Pubs. pp. 413–468

Laskowski D.A. 2002. Physical and chemical properties of pyrethroids. Revue of Environmental Contamination and toxicology 174: 49-170.

Lamoureux G.L., Rusness D.G. 1989. The role of Glutathione and Glutahione-S-Transferases in Pesticide Metabolism, Selectivity, and Mode of Action in Plants and Insects. Coenzymes and Cofactors - John Wiley & Sons p. 153-196

Lee S., Gan J., Kabashima J. 2002. Recovery of synthetic pyrethroids in water samples during storage and extraction. Journal of agricultural and food chemistry 50: 7194-7198

Liu M-Y., Casida J.E. 1993. High affinity binding of [^{3}H] imidacloprid in the insect acetylcholine receptor. Pesticide biochemistry and physiology 46: 40-46

Lozano S.J., O'Halloran S.L. Sargent K.W., Brazner J.C. 1992. Effects of esfenvalerate on aquatic organisms in littoral enclosures. Environmental Toxicology and Chemistry 11: 35-47

Malato S., Caceres J., Aguera A., Mezcua M., Hernando D., Vial J., Fernandez-Alba A.R. 2001. Degradation of imidacloprid in Water by photo-fenton and TiO_2 photocatalysis at a solar pilot plant: A comparative study. Environmental science and technology 35: 4359-4366

Maltby L. 1995. Sensitivity of the crustaceans *Gammarus pulex* (L.) and *Asellus aquaticus* (L.) to short-term exposure to hypoxia and unionized ammonia: observations and possible mechanisms. Water research 29 (3): 781-787

Martin T.R., Holdich D.M.1986. The acute lethal toxicity of heavy metals to peracarid crustaceans (with particular. reference to freshwater asellids and gammarids). Water Research 20 (9): 1137-1147

Matoničkin I. 1981. Beskralješnjaci, Biologija viših avertebrata. II izdanje, Školska knjiga, Zagreb. bp 280-314

McCahon C.P., Pascoe D. 1988. Culture techniques for three freshwater macroinvertebrate species and their use in toxicity tests Chemosphere 17(12): 2471-2480

Migliore L., De Nicola Giudici M. 1990. Toxicity of heavy metals to *Asellus aquaticus* (L.) (Crustacea, Isopoda). Hydrobiologia 203 (3): 155-164

MOP - Ministrstvo za okolje in prostor. Hidrometeorološki zavod RS. 2000. Poročilo o monitoringu kakovosti podtalnice in površinskih vodotokov na območju mestne občine Ljubljana v letu 1999/2000

Moza P.N., Hustert K., Feicht E., Kettrup A. 1998. Photolysis of imidacloprid in aqueous solution. Chemosphere 36 (3): 497-502

Mulliss R., Ellis J.B., Revitt D.M., Shutes R.B.E. 1994. An evaluation of the toxic influences on *Asellus aquaticus* (L) in an urban stream environment. Water Science and Technology 29 (1-2): 199-207

Mulliss R.M., Revitt D.M., Shutes R.B.E. 1996. The determination of the toxic influences to *Gammarus pulex* (amphipoda) caged in urban receiving waters. Ecotoxicology 5 (5): 209-215

Munn M.D., Gillom R.J. 2001. Pesticide Toxicity Index for Freshwater Aquatic Organisms. U.S. Geological Survey. Water-Resources Investigations Report 01 – 4077 National water-quality assessment program

Musko I.B., G.-Tóth L., Szabo E. 1995. Respiration and respiratory electron transport system (ETS) activity of two amphipods: *Corophium curvispinum* G.O.Sars and *Gammarus fossarum* Koch. Polskie archiwum hydrobiologii 42 (4): 547-558

Naylor C., Maltby L., Calow P. 1990. Inter-and intra-specific variation in sensitivity to toxins; the effects of acidity and zinc on the freshwater crustaceans *Asellus aquaticus* (L.) and *Gammarus pulex* (L.). Water research 24: 757-762

Nielsen S.A., Jensen K.M.V., Kristensen M., Westh P. 2006. Energetic Costs of Subacute Chlorpyrifos Intoxication in the German Cockroach (Dictyoptera: Blattelliadae). Physiological Ecology 35(4): 837-842

Nielsen S.A., Toft S., Clausen J. 1999. Cypermethrin effects on detoxification enzymes in active and hibernating wolf spider (*Pardosa amentata*). Ecological Applications 9(2): 463-468

Official journal of RS no. 29/1996 from 31.05.1996 i.p. address:
http://www.uradni-list.si/1/objava.jsp?urlid=199629&stevilka=1843 (01.03.2008)

Owens T.G., King F.D. 1975. The measurement of respiratory electron-transport-system activity in marine zooplankton. Marine biology 30 (1): 27-36

Pan-Europe, 2000. Joint PAN Europe and Ecological Council. Briefing on esfenvalerate.
i.p. address: http://www.pan-europe.info/Resources/Policy/Archive/esfen.pdf (10.04.2008)

Packard T.T. 1971. The measurement of respiratory electron-transport activity in marine phytoplankton. Journal of marine research 29: 235-244

Pantani C., Pannunzio G., De Cristfaro M., Novelli A.A., Slavatori M. 1997. Comparative acute toxicity of some pesticides, metals and surfactants to *Gammarus italicus* Goedm. And *Echinogammrus tibaldii* Pink. and Stock (Crustacea: Amphipoda). Bulletin of Environmental Contamination and Toxicology 59 (6): 963-967

Pascoe D., Carroll K. 2004. Comparison of cadmium toxicity to *Asellus aquaticus* (L.) populations following 17 years isolation in pond and laboratory cultures. Bulletin of Environmental Contamination and Toxicology : 73: 167-173

Pascoe D., Kedwards T.J., Blockwell S.J., Taylor E.J. 1995. *Gammarus pulex* (L) Feeding bioassay – Effects of Parasitism. Bulletin of Environmental Contamination and Toxicology 55: 629-632

Peters R.H. 1987. Metabolism in *Daphnia* Memorie dell'Istituto Italiano di Idrobiologia 45: 193-243

Philippova T.G., Postnov A.L. 1988. The effect of food quantity on feeding and metabolic expenditure in Cladocera. International Revue of Gesamten Hydrobiology 73 (6): 601-615

Pierce, 2003. Instructions for BCA Protein Assay Kit. Product numbers 23225 and 23227. Pierce Biotehnology, Inc., 10/2003. Printed in the USA

Plaistow S.J., Bollache L., Cezilly F. 2003. Energetically costly precopulatory mate guarding in the amphipod Gammarus pulex: causes and consequences. Animal behaviour 65: 683-691

Plenet S. 1995. Freshwater amphipods as biomonitors of metal pollution in surface and interstitial aquatic systems. Freshwater biology 33:127-137

Pöckl M. 1993. Reproductive potential and lifetime potential fecundity of the freshwater amphipods *Gammarus fossarum* and *G. roeseli* in Austrian streams and rivers. Freshwater Biology 30: 73-91

Prohaska J.R. 1980. The glutathione peroxidase activity of glutathione S-transferases. Biochimica et Biophysica Acta 611: 87-98

Qiao X., Ma L., Hummel H.E. 1996. Persistence of atrazine and occurrence of its primary metabolites in three soils. Journal of agricultural and food chemistry 44: 2846-2848

Randall D, Burggren W, French K, 2000. Eckert animal physiology: mechanisms and adaptations Fourth edition, USA. P.676-680

Richardson S.D. 2007. Water analysis: Emerging contaminants and current issues. Analytical chemistry 79: 4295-4324

Richardson S.D., Ternes T.A. 2005. Water analysis: Emerging contaminants and current issues. Analytical chemistry 77: 3807-3838

Rinderhagen M., Ritterhoff J., Zauke G.P. 2000. Crustaceans as Bioindicators. Carl von Ossietzky Universität Oldenburg, FB Biologie (ICBM), Oldenburg, Germany

Ritter WF, Scarborough RW, Chirnside AEM, 1994. Contamination of groundwater by triazines, metolachlor and alachlor*. Journal of contaminant hydrology 15: 73-92

Ruppert E.E., Fox R.S., Barnes R.D. 2004. Crustacea. Intervebrate zoology. A functional evolutionary approach. Seventh edition, Thomson Learning, London, United Kingdom p.(19) 605-701

Sánchez-Bayo F., Goka K. 2006. Influence of light in acute toxicity bioassays of imidacloprid and zinc pyrithione to zooplankton crustaceans. Aquatic toxicology 78 (3): 262-271

Sarkar M.A., Biswas P.K., Roy S., Kole R.K., Chowdhury A. 1999. Effect of pH and type of formulation on the persistence of Imidacloprid in water. Bulletin of Environmental Contamination and Toxicology 63: 604-609

Savenkoff C., da Silva N.L., Lefèvre D., Denis M., Rassoulzadegan F. 1992. Contribution of the different planktonic microbial assemblages to ETS activity in the Ligurian frontal area: north-west Mediterranean Sea. Journal of plankton research 14 (6): 835-850

Schmidt-Nielsen K. 1979. Animal physiology. Adaptation and environment. Second edition Cambridge university press

Selye H. 1975. The Stress of Life. 2nd edition. McGraw-Hill. New York City

Sherma J. 1993. Pesticides. Analytical chemistry 65: 40R-54R

Systat software; SigmaStat 3.5 - Advisory Statistics for Science and Industry i.p.: http://www.systat.com/products/SigmaStat/productinfo/?sec=1003 (12.03.2008)

Simčič T., Brancelj A. 1997. Electron transport system (ETS) activity and respiration rate in five Daphnia species at different temperatures Hydrobiologia 360: 117-125

Simčič T., Brancelj A. 2000. En energy exploitation in *Chirocephalus croaticus* (Steuer, 1899) (Crustacea : Anostraca): survival strategy in an intermittent lakes Hydrobiologia 437: 157-163

Simčič T., Brancelj A. 2001. Seasonal dynamics of metabolic activity of the *Daphnia* community in Lake Bled (Slovenia). Hydrobiologia 442: 319-328

Simčič T., Brancelj A. 2003. Estimation of the proportion of metabolically active mass in the amphipod Gammarus fossarum Freshwater Biology 48 (6): 1093-1099

Simčič T., Lukančič S., Brancelj A. 2005. Comparative study of electron transport system activity and oxygen consumption of amphipods from caves and surface habitats Freshwater Biology 50: 494-501

Sket B., Gogala M., Kuštor V. 2003. Živalstvo Slovenije 1. natis, Tehniška založba Slovenije, Ljubljana

Solomon K.R., Baker D.B., Richards R.P., Dixon K.R., Klaine S.J., La Point T.W., Kendall R.J., Weisskopf C.P., Giddings J.G., Giesy J.P., Hall L.W., Williams W.M. 1996. Ecological risk assessment of atrazine in North American surface waters. Environmental Toxicology and Chemistry 15 (1): 31-76

Song M.Y., Stark J.D., Brown J.J. 1997. Comparative toxicity of four insecticides, including Imidacloprid and Tebufenozide, to four aquatic arthropods. Environmental Toxicology and Chemistry 16 (12): 2494-2500

Stark J.D., Banks J.E. 2001. "Selective" pesticides: Are they less hazardous to the environment. Bioscience 51 (11): 480-482

Stenersen J., Kobro S., Bjerke M., Arend U. 1987. Glutathione transferases in aquatic and terrestrial animals from nine phyla. Comparative biochemistry and Physiology 86C: 73-82

Storey K.B. 1996. Oxidative stress: animal adaptations in nature. Brazilian Journal of Medical and Biological research 29: 1715-1733

Tomizawa M., Casida J.E. 2003. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annual revue of entomology 48: 339-364.

Van Gestel C.A.M., Brummelen T.C. 1996. Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. ECOTOXIOLOGY 5: 217-225

Voet D., Voet J.G. 2006. Biochemistry. John Wiley & Sons, Inc., p. 528-560

Vontas J.G., Small G.J., Hemingway J. 2001. Glutathione S-transferases as antioxidant defense agents confer pyrethroid resistance in *Nilaparvata lugens*. Journal of Biochemistry 357: 65-72

Walker C.H., Hopkin S.P., Sibly R.M., Peakall D.B. 1996. Principles of ecotoxicology. London, Taylor & Francis bp 321

Ware G.W., Whitacre D.M. 2004. An introduction to insecticides. Extracted from The Pesticide Book, 6th edition, published by MeisterPro Information Resources Adivision of Meister Media Worldwide, Willoughby, Ohio. i.p.adress: http://ipmworld.umn.edu/chapters/ware.htm (22.01.2008)

Wauchope R.D., Buttler T.M., Hornsby A.G., Augustijn-Beckers P.W.M. and Burt J.P. 1992. Pesticides properties database for environmental decisionmaking. Revue of Environmental Contamination and Toxicololology 123: 2-26

Wijnhoven S., van Riel M.C., Van der Velde G. 2003. Exotic and indigenous freshwater gammarid species: physiological tolerance to water temperature in relation to ionic content of the water. Aquatic ecology 37(2): 151-158

Williams K.A., Green D.W.J., Pascoe D. 1985. Studies on the Acute Toxicity of Pollutants to Freshwater Macroinvertebrates; 1. Cadmium Archive fur Hydrobiologie 102 (4): 461-471

Yurista P.M. 1999. Temperature-dependent energy budget of an Arctic Cladoceran, *Daphnia middendorffiana.* Freshwater biology 42: 21-34

Zheng W., Liu W. 1999. Kinetics and mechanism of the hydrolysis of imidacloprid. Pesticide science 55: 482-485

Appendix 1: Descriptive Statistics **WW/DW *for A. aquaticus* – size difference**

Data source: WW/DW for *A. aquaticus*

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
WW/DW	20-45	11	0	4.938	1.015	0.306	0.682
WW/DW	<20	9	0	6.654	1.773	0.591	1.363

Column	Range	Max	Min	Median	25%	75%
WW/DW	20-45	2.983	6.743	3.760	4.571	4.216	5.845
WW/DW	<20	5.269	9.000	3.731	6.571	5.405	8.362

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
WW/DW	20-45	0.751	-0.732	0.203	0.228	54.317	278.517
WW/DW	<20	-0.269	-0.850	0.159	0.635	59.887	423.641

Data source: WW/DW for *A. aquaticus*

Normality Test: Passed (P = 0.442)

Equal Variance Test: Passed (P = 0.093)

Group Name	N	Missing	Mean	Std Dev	SEM
WW/DW	20-45	11	0	4.938	1.015	0.306
WW/DW	<20	9	0	6.654	1.773	0.591

Difference -1.716

t = -2.721 with 18 degrees of freedom. (P = 0.014)

95 percent confidence interval for difference of means: -3.041 to -0.391

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.014).

Power of performed test with alpha = 0.050: 0.671

Appendix 2: Descriptive Statistics **WW/DW *for G. fossarum* – size difference**

Data source: WW/DW for *G. fossarum*

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
WW/DW 20-45	11	0	4.709	0.731	0.220	0.491
WW/DW <20	13	0	4.150	0.339	0.0941	0.205

Column	Range	Max	Min	Median	25%	75%
WW/DW 20-45	2.341	5.803	3.463	4.659	4.380	5.294
WW/DW <20	1.250	4.762	3.512	4.115	4.045	4.405

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
WW/DW 20-45	-0.290	-0.697	0.143	0.663	51.794	249.214
WW/DW <20	-0.308	0.259	0.203	0.150	53.956	225.319

Data source: WW/DW for *G. fossarum*

Normality Test: Passed (P = 0.396)

Equal Variance Test: Failed (P < 0.050)

Group	N	Missing	Median	25%	75%
WW/DW 20-45	11	0	4.659	4.380	5.294
WW/DW <20	13	0	4.115	4.045	4.405

Mann-Whitney U Statistic= 37.000

T = 172.000 n(small)= 11 n(big)= 13 (P = 0,049)

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = 0.049)

Appendix 3: Descriptive Statistics **ETS/R ratio *for A. aquaticus* at two different temperatures**

Data source: *Asellus aquaticus* difference in temperature

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
10 °C Iščica	32	0	3.652	1.060	0.187	0.382
10 °C Cerkniško	25	0	5.289	1.451	0.290	0.599
15 °C Cerkniško	13	0	8.755	1.367	0.379	0.826

Column	Range	Max	Min	Median	25%	75%
10 °C Iščica	4.459	6.140	1.681	3.402	2.930	4.251
10 °C Cerkniško	4.730	8.209	3.479	4.721	4.144	6.370
15 °C Cerkniško	4.436	11.407	6.971	8.631	7.515	9.674

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
10 °C Iščica	0.524	-0.115	0.143	0.094	116.864	461.648
10 °C Cerkniško	0.710	-0.891	0.213	0.005	132.231	749.921
15 °C Cerkniško	0.257	-0.588	0.124	0.733	13.816	1018.913

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: *Asellus aquaticus* diff temperature

Normality Test: Failed (P < 0.050)

Group	N	Missing	Median	25%	75%
10 °C Iscica	32	0	3.402	2.930	4.251
10 °C Cerkniško	25	0	4.721	4.144	6.370
15 °C Cerkniško	13	0	8.631	7.515	9.674

H = 41.839 with 2 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pair wise Multiple Comparison Procedures (Dunn's Method):

Comparison	Diff of Ranks	Q	P<0.05
15 °C Cerkniško vs 10 °C Iščica	42.053	6.283	Yes
15 °C Cerkniško vs 10 °C Cerkniško	22.095	3.175	Yes
10 °C Cerkniško vs 10 °C Iščica	19.958	3.674	Yes

Appendix 2: Descriptive Statistics **ETS/R ratio *for G. fossarum* at two different temperatures**

Descriptive Statistics:

Data source: *G. fossarum* difference in the temperature

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
10 °C Duplje	17	0	4.661	1.345	0.326	0.692
10 °C Iscica	33	0	2.587	0.835	0.145	0.296
15 °C Iscica	8	0	3.928	1.275	0.451	1.066

Column	Range	Max	Min	Median	25%	75%
10 °C Duplje	4.553	6.977	2.425	4.186	3.789	5.937
10 °C Iscica	3.569	4.404	0.835	2.641	2.034	3.276
15 °C Iscica	3.365	5.639	2.274	3.586	2.980	5.187

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
10 °C Duplje	0.289	-0.693	0.167	0.227	79.244	398.349
10 °C Iscica	-0.0366	-0.328	0.0647	0.875	85.380	243.231
15 °C Iscica	0.138	-1.569	0.208	0.361	31.420	134.774

One Way Analysis of Variance

Data source: *G. fossarum* difference in the temperature

Normality Test: Passed(P = 0.455)

Equal Variance Test: Passed(P = 0.097)

Group Name	N	Missing	Mean	SD	SEM
10 °C Duplje	17	0	4.661	1.345	0.326
10 °C Iscica	33	0	2.587	0.835	0.145
15 °C Iscica	8	0	3.928	1.275	0.451

Source of Variation	DF	SS	MS	F	P
Between Groups	2	51.051	25.525	22.406	<0.001
Residual	55	62.656	1.139		
Total	57	113.706			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pair wise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
10 °C Duplje vs. 10 °C Iscica	2.074	6.509	2.39	0.017	Yes
15 °C Iscica vs. 10 °C Iscica	1.340	3.186	2.38E-3	0.025	Yes
10 °C Duplje vs. 15 °C Iscica	0.734	1.604	0.114	0.050	No

Appendix 3: Descriptive Statistics **ETS/R ratio for *A. aquaticus* in copper sulfate**

Data source: Data in copper sulfate Aa 3 conc

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
Control	5	0	4.120	1.011	0.452	1.256
3 mg l^{-1}	8	0	4.171	0.856	0.303	0.716
5 mg l^{-1}	8	0	3.954	0.971	0.343	0.812
10 mg l^{-1}	5	0	3.207	0.846	0.379	1.051

Column	Range	Max	Min	Median	25%	75%
Control	2.657	5.733	3.076	3.817	3.483	4.702
3 mg l^{-1}	2.174	5.176	3.002	4.454	3.296	4.844
5 mg l^{-1}	2.780	4.977	2.197	4.380	3.255	4.594
10 mg l^{-1}	1.976	4.122	2.147	3.087	2.550	4.025

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
Control	1.172	1.586	0.218	0.512	20.602	88.978
3 mg l^{-1}	-0.456	-1.695	0.191	0.472	33.366	144.288
5 mg l^{-1}	-1.003	-0.115	0.276	0.073	31.632	131.675
10 mg l^{-1}	-0.0567	-2.106	0.223	0.484	16.033	54.275

One Way Analysis of Variance

Data source: Data in copper sulfate Aa 3 conc
Normality Test: Passed(P = 0.405)
Equal Variance Test: Passed(P = 0.965)
Analyses of variance passed. Normal distribution.

Group Name	N	Missing	Mean	SD	SEM
Control	5	0	4.120	1.011	0.452
3 mg l^{-1}	8	0	4.171	0.856	0.303
5 mg l^{-1}	8	0	3.954	0.971	0.343
10 mg l^{-1}	5	0	3.207	0.846	0.379

Source of Variation	DF	SS	MS	F	P
Between Groups	3	3.255	1.085	**1.277**	**0.307**
Residual	22	18.688	0.849		
Total	25	21.943			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.307).
Power of performed test with alpha = 0.050: 0.093
The power of the performed test (0.093) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

t-test

Data source: Data in Stat copper sulfate Aa 3+1
Normality Test: Passed(P = 0.649)
Equal Variance Test: Passed(P = 0.946)

Group Name	N	Missing	Mean	SD	SEM
Control	5	0	4.120	1.011	0.452
10 mg l^{-1}	5	0	3.207	0.846	0.379
Difference	0.914				

t = 1.549 with 8 degrees of freedom. (**P = 0.160**)
95 percent confidence interval for difference of means: -0.446 to 2.274
The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.160).
Power of performed test with alpha = 0.050: 0.174
The power of the performed test (0.174) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

Appendix 4: Descriptive Statistics **ETS/R ratio for *A. aquaticus* in imidacloprid**

Data source: Data in Stat ETS/R imidacloprid Aa

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
Contr	9	0	2.166	0.527	0.176	0.405
0.01 mg l^{-1}	24	0	1.963	0.423	0.0863	0.178
0.1 mg l^{-1}	30	0	2.193	0.631	0.115	0.236
1 mg l^{-1}	26	0	1.571	0.404	0.0792	0.163
10 mg l^{-1}	30	0	1.272	0.293	0.0535	0.109

Column	Range	Max	Min	Median	25%	75%
Contr	1.298	3.065	1.767	1.880	1.778	2.557
0.01 mg l^{-1}	1.674	2.862	1.188	1.961	1.691	2.307
0.1 mg l^{-1}	2.349	3.395	1.046	2.088	1.766	2.634
1 mg l^{-1}	1.506	2.444	0.938	1.497	1.282	1.803
10 mg l^{-1}	1.221	2.018	0.797	1.215	1.092	1.373

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
Contr	1.130	-0.444	0.278	0.044	19.496	44.456
0.01 mg l^{-1}	0.0805	-0.398	0.104	0.651	47.104	96.559
0.1 mg l^{-1}	0.352	-0.672	0.109	0.459	65.805	155.879
1 mg l^{-1}	0.463	-0.250	0.099	0.662	40.856	68.278
10 mg l^{-1}	0.809	0.586	0.132	0.195	38.158	51.021

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data in Stat imidacloprid Aa
Normality Test: Passed (P = 0.059)
Equal Variance Test: Failed (P < 0.050)

Group	N	Missing	Median	25%	75%
Contr	9	0	1.880	1.778	2.557
0.01 mg l^{-1}	24	0	1.961	1.691	2.307
0.1 mg l^{-1}	30	0	2.088	1.766	2.634
1 mg l^{-1}	26	0	1.497	1.282	1.803
10 mg l^{-1}	30	0	1.215	1.092	1.373

H = 51.053 with 4 degrees of freedom. (**P = <0.001**)
The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (**P = <0.001**)
To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pair wise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
Control vs 10 mg l^{-1}	57.322	4.372	Yes
Control vs 1 mg l^{-1}	35.697	2.676	Yes
Control vs 0.01 mg l^{-1}	10.472	0.777	No
Control vs 0.1 mg l^{-1}	2.089	0.159	No
0.1 mg l^{-1} vs 10 mg l^{-1}	55.233	6.201	Yes
0.1 mg l^{-1} vs 1 mg l^{-1}	33.608	3.636	Yes
0.1 mg l^{-1} vs 0.01 mg l^{-1}	8.383	0.887	No
0.01 mg l^{-1} vs 10 mg l^{-1}	46.850	4.959	Yes
0.01 mg l^{-1} vs 1 mg l^{-1}	25.224	2.583	Yes
1 mg l^{-1} vs 10 mg l^{-1}	21.626	2.340	Yes Mann-Whitney confirmed

Additional One Way Analysis of Variance for three groups 0.01, 1 and 10 mg l^{-1}

Data source: Data in Stat ETS/R imidacloprid for *A. aquaticus*

Group Name	N	Missing	Mean	SD	SEM
0.01 mg l^{-1}	24	0	1.963	0.423	0.0863
1 mg l^{-1}	26	0	1.571	0.404	0.0792
10 mg l^{-1}	30	0	1.272	0.293	0.0535

Source of Variation	DF	SS	MS	F	P
Between Groups	2	6.363	3.181	**22.954**	**<0.001**
Residual	77	10.672	0.139		
Total	79	17.034			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (**P = <0.001**).
Power of performed test with alpha = 0.050: 1.000

All Pair wise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05
Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
0.01 mg l^{-1} vs. 10 mg l^{-1}	0.691	6.775	2.210E-9	0.017	Yes
0.01 mg l^{-1} vs. 1 mg l^{-1}	0.391	3.713	3.850E-4	0.025	Yes
1 mg l^{-1}vs. 10 mg l^{-1}	0.299	3.002	3.61E-3	0.050	Yes

Mann-Whitney Rank Sum Test

Data source: Data in Stat imidacloprid Aa

Group	N	Missing	Median	25%	75%
0.1 mg l^{-1}	24	0	1.961	1.691	2.307
10 mg l^{-1}	30	0	1.215	1.092	1.373

Mann-Whitney U Statistic= 65.000
T = 955.000 n(small)= 24 n(big)= 30 (P = <0.001)
The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

Appendix 5: Descriptive Statistics **ETS/R ratio for *Gammarus* in imidacloprid**

Data source: Data in Stat imidacloprid Gf

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
Control	11	0	4.364	1.371	0.413	0.921
0.01 mg l^{-1}	25	0	2.891	0.638	0.128	0.263
0.1 mg l^{-1}	21	0	3.593	0.940	0.205	0.428
10 mg l^{-1}	18	0	2.633	0.822	0.194	0.409

Column	Range	Max	Min	Median	25%	75%
Control	4.342	6.767	2.425	4.111	3.671	5.631
0.01 mg l^{-1}	2.469	4.281	1.813	2.833	2.427	3.310
0.1 mg l^{-1}	3.539	5.688	2.149	3.486	2.857	3.912
10 mg l^{-1}	2.207	3.932	1.725	2.330	1.944	3.478

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
Control	0.433	-0.624	0.188	0.323	48.006	228.301
0.01 mg l^{-1}	0.493	-0.193	0.144	0.197	72.278	218.735
0.1 mg l^{-1}	0.890	0.419	0.159	0.176	75.461	288.837
10 mg l^{-1}	0.583	-1.258	0.179	0.129	47.400	136.300

Kruskal-Wallis One Way Analysis of Variance on Ranks
Data source: Data in Stat imidacloprid Gf

Normality Test: Failed (P < 0.050)

Group	N	Missing	Median	25%	75%
Control	11	0	4.111	3.671	5.631
0.01 mg l^{-1}	25	0	2.833	2.427	3.310
0.1 mg l^{-1}	21	0	3.486	2.857	3.912
10 mg l^{-1}	18	0	2.330	1.944	3.478

H = 19.721 with 3 degrees of freedom. (**P = <0.001**)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (**P = <0.001**)
To isolate the group or groups that differ from the others use a multiple comparison procedure.
All Pair wise Multiple Comparison Procedures (Dunn's Method):

Comparison	Diff of Ranks	Q	P<0.05
Control vs 10 mg l^{-1}	31.485	3.775	Yes
Control vs 0.01 mg l^{-1}	25.218	3.198	Yes
Control vs 0.1 mg l^{-1}	10.199	1.257	No
0.1 mg l^{-1} vs 10 mg l^{-1}	21.286	3.041	Yes
0.1 mg l^{-1}vs 0.01 mg l^{-1}	15.019	2.328	No
0.01 mg l^{-1} vs 10 mg l^{-1}	6.267	0.930	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Appendix 6: Descriptive Statistics **ETS/R ratio for *A. aquaticus* in atrazine**

Data source: Data in Stat atrazine Aa

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
Control	9	0	9.313	2.409	0.803	1.852
5 mg l^{-1}	10	0	8.450	2.389	0.756	1.709
10 mg l^{-1}	9	0	4.284	1.588	0.529	1.221

Column	Range	Max	Min	Median	25%	75%
Control	7.337	13.434	6.096	9.292	7.437	11.266
5 mg l^{-1}	7.709	12.108	4.399	7.566	7.182	10.282
10 mg l^{-1}	4.623	7.300	2.677	3.847	3.067	5.058

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
Control	0.390	-0.856	0.205	0.318	83.819	827.038
5 mg l^{-1}	0.118	-0.519	0.225	0.161	84.502	765.436
10 mg l^{-1}	1.027	0.163	0.186	0.447	38.558	185.379

One Way Analysis of Variance

Data source: Data in Stat atrazine Aa

Group Name	N	Missing	Mean	SD	SEM
Control	9	0	9.313	2.409	0.803
5 mg l^{-1}	10	0	8.450	2.389	0.756
10 mg l^{-1}	9	0	4.284	1.588	0.529

Source of Variation	DF	SS	MS	F	P
Between Groups	2	131.337	65.668	**13.914**	**<0.001**
Residual	25	117.987	4.719		
Total	27	249.324			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (**P = <0.001**).

Power of performed test with alpha = 0.050: 0.996

All Pair wise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Control vs. 10 mg l^{-1}	5.029	4.911	4.700E-5	0.017	Yes
5 mg l^{-1} vs. 10 mg l^{-1}	4.166	4.174	3.170E-4	0.025	Yes
Control vs. 5 mg l^{-1}	0.863	0.865	0.395	0.050	No

Appendix 7: Descriptive Statistics **ETS/R ratio for *G. fossarum* in atrazine**

Data source: Data in Stat atrazine Gf

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
Control	18	0	6.761	1.677	0.395	0.834
1 mg l^{-1}	21	0	4.713	1.647	0.359	0.750
3 mg l^{-1}	22	0	4.284	1.383	0.295	0.613
10 mg l^{-1}	18	0	4.944	1.047	0.247	0.521

Column	Range	Max	Min	Median	25%	75%
Control	5.385	9.476	4.090	6.970	5.041	8.320
1 mg l^{-1}	6.162	7.923	1.760	4.708	3.525	5.910
3 mg l^{-1}	5.396	7.367	1.971	4.349	3.140	5.235
10 mg l^{-1}	4.153	6.844	2.691	4.964	4.460	5.589

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
Control	-0.171	-1.095	0.125	0.580	121.696	870.563
1 mg l^{-1}	0.0111	-0.501	0.0750	0.866	98.980	520.789
3 mg l^{-1}	0.275	-0.320	0.0942	0.782	94.242	443.869
10 mg l^{-1}	-0.335	0.221	0.0997	0.799	88.996	458.656

Analyses of variance passed. Normal distribution.

One Way Analysis of Variance

Data source: Data in Stat atrazine Gf

Group Name	N	Missing	Mean	SD	SEM
Control	18	0	6.761	1.677	0.395
1 mg l^{-1}	21	0	4.713	1.647	0.359
3 mg l^{-1}	22	0	4.284	1.383	0.295
10 mg l^{-1}	18	0	4.944	1.047	0.247

Source of Variation	DF	SS	MS	F	P
Between Groups	3	67.878	22.626	**10.549**	**<0.001**
Residual	75	160.861	2.145		
Total	78	228.739			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (**P = <0.001**).

Power of performed test with alpha = 0.050: 0.998

All Pair wise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Control vs. 3 mg l^{-1}	2.477	5.322	1.030E-6	0.009	Yes
Control vs. 1 mg l^{-1}	2.048	4.353	4.190E-5	0.010	Yes
Control vs. 10 mg l^{-1}	1.817	3.721	3.810E-4	0.013	Yes
10 mg l^{-1} vs. 3 mg l^{-1}	0.661	1.419	0.160	0.017	No
1 mg l^{-1} vs. 3 mg l^{-1}	0.430	0.962	0.339	0.025	No
10 mg l^{-1} vs. 1 mg l^{-1}	0.231	0.491	0.625	0.050	No

Appendix 8: Descriptive Statistics for calorimetric measurements lnDW in esfenvalerate

Data source: Data in Denmark Gf calorimeter

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
Control	13	0	0.656	0.197	0.0547	0.119
0.02 µg l^{-1}	13	0	0.990	0.146	0.0405	0.0881
0.1 µg l^{-1}	16	0	1.155	0.276	0.0690	0.147
0.2 µg l^{-1}	16	0	1.796	0.642	0.161	0.342
0.3 µg l^{-1}	15	0	4.329	1.219	0.315	0.675

Column	Range	Max	Min	Median	25%	75%
Control	0.567	0.913	0.346	0.696	0.525	0.841
0.02 µg l^{-1}	0.593	1.273	0.680	0.959	0.920	1.069
0.1 µg l^{-1}	0.851	1.672	0.820	1.062	0.942	1.420
0.2 µg l^{-1}	2.005	2.978	0.973	1.624	1.334	2.024
0.3 µg l^{-1}	2.904	5.876	2.972	4.244	3.054	5.565

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
Control	-0.414	-1.145	0.151	0.525	8.533	6.069
0.02 µg l^{-1}	-0.211	1.133	0.149	0.535	12.865	12.986
0.1 µg l^{-1}	0.505	-1.167	0.182	0.167	18.478	22.482
0.2 µg l^{-1}	0.951	-0.153	0.163	0.293	28.735	57.789
0.3 µg l^{-1}	0.0741	-1.908	0.238	0.022	64.930	301.872

Kruskal-Wallis One Way Analysis of Variance on Ranks
Data source: Data in Denmark Gf calorimeter
Normality Test: **Failed (P < 0.050)**

Group	N	Missing	Median	25%	75%
Control	13	0	0.696	0.525	0.841
0.02 µg l^{-1}	13	0	0.959	0.920	1.069
0.1 µg l^{-1}	16	0	1.062	0.942	1.420
0.2 µg l^{-1}	16	0	1.624	1.334	2.024
0.3 µg l^{-1}	15	0	4.244	3.054	5.565

H = 59.956 with 4 degrees of freedom. (P = <0.001)
The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)
To isolate the group or groups that differ from the others use a multiple comparison procedure.
All Pair wise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
0.3 µg l^{-1} vs. Control	57.395	7.139	Yes
0.3 µg l^{-1} vs. 0.02 µg l^{-1}	40.395	5.024	Yes
0.3 µg l^{-1} vs. 0.1 µg l^{-1}	34.121	4.475	Yes
0.3 µg l^{-1} vs. 0.2 µg l^{-1}	18.433	2.417	No
0.2 µg l^{-1} vs. Control	38.962	4.918	Yes
0.2 µg l^{-1} vs. 0.02 µg l^{-1}	21.962	2.772	Yes
0.2 µg l^{-1} vs. 0.1 µg l^{-1}	15.688	2.091	Do Not Test –> Mann-Whitney

Mann-Whitney Rank Sum Test
Data source: Data in Denmark Gf calorimeter
Normality Test: Passed (P = 0.094)
Equal Variance Test: **Failed (P < 0.050)**

Group	N	Missing	Median	25%	75%
0.1 µg	16	0	1.062	0.942	1.420
0.2 µg	16	0	1.624	1.334	2.024

Mann-Whitney U Statistic= 216000
T = 176000 n(small)= 16 n(big)= 16 (**P = <0.001**)

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (**P = <0.001**).

Appendix 9: Descriptive Statistics for Stress enzymes GST in esfenvalerate
Descriptive Statistics: One Way Analysis of Variance
Data source: Data in Denmark Stress enzyme GPOx

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
Control	13	0	8.046	0.464	0.129	0.280
0.02 µg l^{-1}	18	0	8.186	0.450	0.106	0.224
0.1 µg l^{-1}	18	0	8.211	0.298	0.0703	0.148
0.2 µg l^{-1}	18	0	8.230	0.275	0.0648	0.137
0.3 µg l^{-1}	15	0	8.406	0.388	0.100	0.215

Column	Range	Max	Min	Median	25%	75%
Control	1.560	8.555	6.995	8.277	7.845	8.363
0.02 µg l^{-1}	1.851	8.852	7.001	8.311	8.013	8.459
0.1 µg l^{-1}	1.180	8.979	7.799	8.140	7.995	8.326
0.2 µg l^{-1}	1.067	8.672	7.605	8.238	8.074	8.377
0.3 µg l^{-1}	1.221	9.007	7.786	8.346	8.160	8.727

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
Control	-1.119	0.684	0.229	0.060	104.597	844.166
0.02 µg l^{-1}	-1.391	1.873	0.274	<0.001	147.341	1209.515
0.1 µg l^{-1}	1.083	1.376	0.139	0.444	147.806	1215.214
0.2 µg l^{-1}	-0.527	0.260	0.106	0.756	148.147	1220.594
0.3 µg l^{-1}	0.0278	-0.790	0.133	0.605	126.094	1062.079

Analyses of variance passed. Normal distribution.

One Way Analysis of Variance
Data source: Data in Denmark Stress enzyme GPOx

Group Name	N	Missing	Mean	SD	SEM
Control	13	0	8.046	0.464	0.129
0.02 µg l^{-1}	18	0	8.186	0.450	0.106
0.1 µg l^{-1}	18	0	8.211	0.298	0.0703
0.2 µg l^{-1}	18	0	8.230	0.275	0.0648
0.3 µg l^{-1}	15	0	8.406	0.388	0.100

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.939	0.235	**1.654**	**0.169**
Residual	77	10.926	0.142		
Total	81	11.865			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.169).

Power of performed test with alpha = 0.050: 0.200

The power of the performed test (0.200) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

Appendix 10: Descriptive Statistics for Stress enzymes GPOx in esfenvalerate

Data source: Data in Denmark Stress enzymes

Column	Size	Missing	Mean	SD	Std. Error	C.I. of Mean
Control	13	0	2.718	0.488	0.135	0.295
0.02 µg l^{-1}	18	0	2.641	0.435	0.102	0.216
0.1 µg l^{-1}	18	0	3.541	0.249	0.0587	0.124
0.2 µg l^{-1}	18	0	3.709	0.306	0.0722	0.152
0.3 µg l^{-1}	15	0	3.904	0.423	0.109	0.234

Column	Range	Max	Min	Median	25%	75%
Control	1.518	3.266	1.748	2.711	2.422	3.184
0.02 µg l^{-1}	1.603	3.351	1.748	2.697	2.386	3.004
0.1 µg l^{-1}	0.962	4.027	3.065	3.510	3.423	3.721
0.2 µg l^{-1}	1.273	4.436	3.162	3.673	3.469	3.857
0.3 µg l^{-1}	1.575	4.719	3.144	3.929	3.708	4.055

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	Sum	Sum of Squares
Control	-0.767	-0.258	0.156	0.478	35.333	98.895
0.02 µg l^{-1}	-0.430	-0.411	0.141	0.420	47.535	128.745
0.1 µg l^{-1}	0.0111	-0.0000187	0.145	0.386	63.737	226.741
0.2 µg l^{-1}	0.551	0.553	0.116	0.674	66.760	249.195
0.3 µg l^{-1}	0.202	0.363	0.159	0.370	58.557	231.095

Analyses of variance passed. Normal distribution.

One Way Analysis of Variance
Data source: Data in Denmark Stress enzymes

Group Name	N	Missing	Mean	SD	SEM
Control	13	0	2.718	0.488	0.135
0.02 µg l^{-1}	18	0	2.641	0.435	0.102
0.1 µg l^{-1}	18	0	3.541	0.249	0.0587
0.2 µg l^{-1}	18	0	3.709	0.306	0.0722
0.3 µg l^{-1}	15	0	3.904	0.423	0.109

Source of Variation	DF	SS	MS	F	P
Between Groups	4	21.726	5.432	**37.258**	**<0.001**
Residual	77	11.225	0.146		
Total	81	32.951			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = **<0.001**).
Power of performed test with alpha = 0.050: 1.000

All Pair wise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
0.3 µg l^{-1} vs. 0.02 µg l^{-1}	1.263	9.461	1.552E-14	0.005	Yes
0.2 µg l^{-1} vs. 0.02 µg l^{-1}	1.068	8.392	1.787E-12	0.006	Yes
0.3 µg l^{-1} vs. Control	1.186	8.196	4.260E-12	0.006	Yes
0.2 µg l^{-1} vs. Control	0.991	7.130	4.700E-10	0.007	Yes
0.1 µg l^{-1} vs. 0.02 µg l^{-1}	0.900	7.072	6.060E-10	0.009	Yes
0.1 µg l^{-1} vs. Control	0.823	5.922	8.370E-9	0.010	Yes
0.3 µg l^{-1} vs. 0.1 µg l^{-1}	0.363	2.718	8.100E-3	0.013	Yes
0.3 µg l^{-1} vs. 0.2 µg l^{-1}	0.195	1.460	0.148	0.017	No
0.2 µg l^{-1} vs. 0.1 µg l^{-1}	0.168	1.319	0.191	0.025	No
Control vs. 0.02 µg l^{-1}	0.0771	0.555	0.581	0.050	No