CRANFIELD UNIVERSITY

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ELECTROCHEMICAL PEROXIDATION OF CONTAMINATED WATER AND ASSESSMENT OF THE TOXICITY USING EXISTING AND NOVEL BIOASSAYS

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ELECTROCHEMICAL PEROXIDATION OF CONTAMINATED WATER AND ASSESSMENT OF THE TOXICITY USING EXISTING AND NOVEL BIOASSAYS

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ABSTRACT

The treatment of wastewater and monitoring of its toxicity is essential before discharging it to the environment. This study focuses on assessing the toxicity of wastewater following treatment using the electrochemical peroxidation process (ECP). Three categories of low-cost toxicity assays were used based on plant cells, microorganisms and invertebrates, all of which do not require ethical approval or a special licence. In addition, a novel cost-effective device was developed for assessing wastewater toxicity at low concentrations.

In Chapter 2, the problem of high turbidity of the ECP-treated samples is addressed, by accelerating particulate settling of the existent compounds. This was achieved by storing the ECP treated samples at low temperatures. This is an essential step for enhancing the clarity of the processed samples in order to improve the performance of the toxicity assays.

Firstly, the performance of the chloroplast assay was evaluated (Chapter 3). The results showed high sensitivity to atrazine levels below 2×10^{-3} mg/l, which is the maximum allowable concentration according to European regulations. However, this assay did not show the same level of sensitivity to copper ions, phenol and sodium hypochlorite.

The toxic effect of several compounds was also evaluated based on growth inhibition of *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* and bioluminescence deterioration of *Vibrio fischeri* (Chapter 4). The results obtained by the growth inhibition assay showed low sensitivity to most toxic compounds, when tested individually. Furthermore, freshly prepared *V.fischeri* cells proved an unreliable indicator of toxicity due to poor repeatability of results in laboratory conditions.

In Chapter 5, the mortality and the inhibition of mobility of *Artemia salina* nauplii were evaluated and compared. For estimating the mobility of the nauplii, a novel device has been designed and constructed. The optimum conditions of temperature (15-25°C) and the effect of turbidity were determined. This system successfully estimated the average speed per nauplius, which proved to be dependent on the toxicity level of tested samples. Compared to the mortality assay, this device demonstrated higher sensitivity by detecting the existence of toxic compounds at very low concentrations. Additionally, it proved applicable to a wide

range of contaminants including pesticides, pharmaceutical, heavy metal ions and mixtures of contaminants. The toxicity of heavy metal ions was detected below the level indicated by European regulations. The sensitivity of the assay increased when an un-quantified swarm of *Artemia salina* nauplii were used instead of 4 nauplii. In addition, the effectiveness of the device was verified when using compounds proposed by the Environmental Protection Agency. Specifically, aldicarb, colchicine and thallium sulphate showed that the threshold toxicities obtained by the novel device were comparable to ones by Microtox® for the same compounds and significantly lower than the human lethal doses. As a result, the proposed system offers a low-cost alternative to toxicity monitoring.

Finally, in Chapter 6, the aforementioned assays were applied to assess the toxicity of ECP-treated wastewater. The conclusion is that the application of a battery of assays is recommended for assessing the toxicity in wastewater samples since a mixture of contaminants is present.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AOP	Advanced Oxidation Process
ANOVA	Analysis of Variance
ARC	Artemia Reference Centre
ASW	Artificial seawater
ATP	Adenosine Triphosphate
BB3	Basic Blue 3
BOD	Biological Oxidation Demand
BSA	Bovine Serum Albumin
BSI	British Standard Institution
COD	Chemical Oxidation Demand
d.f.	Degrees of freedom
DMF	Dimethylformamide
DO	Direct Orange
DPIP	2,6-dichlorophenolindophenol
EC	Effective Concentration
ECP	Electrochemical Peroxidation
EPA-ETV	Environmental Protection Agency - Environmental Technology Verification
FMN	Flavin mononucleotide
F pr.	F probability
HFO	Hydrous ferric oxides
IBST	Institute of Bioscience and Technology

IC	Inhibitory Concentration
ISO	International organisation for standardisation
LC	Lethal concentration
LED	Light-emitting diode
MAC	Maximum allowable concentration
MOPS	4-morpholinepropanesulfonic acid
m.s.	Mean square
NADP	Nicotinamide adenine dinucleotide phosphate
O.D.	Optical Density
PAS	Publicly Available Specification
PCB	Polychlorinated biphenyl
PS	Photo-system
PVA	Polyvinyl Alcohol
RBB	Reactive Black B
s.s.	Sums of squares
SS	Suspended Solids
SVI	Sludge Volume Index
TSS	Total suspended solid
UV-Vis	Ultraviolet- Visible
US	United States
v	Velocity
v.r.	Variance ratio

Chapter 1 General introduction

1.1 Introduction

As industry has advanced, the resultant increasing volumes of contaminated water have generated strong interest in pre-disposal processing. Wastewater treatment constitutes a major problem since the water resources are diminishing, the wastewater discharge costs continue to rise and the regulations regarding their disposal are becoming stricter. Sources of contaminated water include: compost and landfill leachate; municipal and industrial wastewater; process water from wastewater treatment plants (WWTP); and human waste. Furthermore, untreated wastewater contains stable organic material, toxic compounds and pathogenic microorganisms that threaten the environment and its habitats, their biodiversity and subsequently human health. Thus, there is an imperative need for treating polluted water before its disposal whilst auditing its potential toxicity.

Over the last decades, the electrochemical peroxidation process (ECP) has been established as an effective method for degrading large molecules, which are usually recalcitrant, insoluble and stable for the environment, into smaller molecules, which are more mobile, more soluble but frequently more toxic (Chapter 2). Monitoring the toxicity level of contaminated water before, during and after the ECP process was the main aim of this study. The processed samples included compost leachate and wastewater.

The novelty of this study is based on the combination of the electrochemical peroxidation process (ECP) with a set of toxicity assays. Wastewater samples were treated using the ECP process. The main hypothesis is that the toxicity level of post-treatment wastewater is minimised compared to the toxicity of pre-treatment wastewater. This hypothesis has been investigated using toxicity assays. A wide range of toxicity assays was considered for use with ECP. The application of the most suitable assays was evaluated based on the availability of required substances and cost. The applied assays were based on plant cells - chloroplast assay (Chapter 3), microorganisms - the growth inhibition of *E.coli, P.aeruginosa* and *S.aureus* and the bioluminescence inhibition of *V.fischeri* (Chapter 4) and invertebrates -

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the mortality and the mobility assessment of *Artemia salina* nauplii (Chapter 5). These include the three main categories of toxicity assays that do not require ethics approval. Furthermore, as part of this study a newly developed device is presented that monitors the toxicity level in aqueous solutions using *Artemia salina* nauplii. This approach is performed for the first time by combining the ECP wastewater remediation process with low-cost toxicity assays (Chapter 6). In the following sections an overview of the background of the project will be presented.

1.2   Composting

Composting is a process for treating waste and can be divided into industrial composting and home composting. Although the same biological treatments are carried out, they differentiate via several factors and techniques. Composting has beneficial results, such as decreasing the quantities of biodegradable municipal waste. Furthermore, its products are usable for horticulture and agriculture as fertilizers and as nutrient providers for improving the quality of the soil.

Composting consists of four stages:

- Firstly, the *mesophilic phase*, where the mesophilic bacteria propagate and raise the temperature to 44°C. Due to continuously raising temperatures exist in the composting mass (44-52°C), the mesophilic bacteria cannot survive and are replaced by the thermophilic bacteria.
- The second phase, *thermophilic phase*, starts when the activity of the thermophilic bacteria is rising and they are producing high amounts of heat. The temperature can reach the 70°C. During this phase, the thermophilic microorganisms decompose fats, lipids and proteins. However, some other microorganisms, such as fungi, degrade cellulose, lignin and other complex organic compounds.
- The third phase called *cooling phase*, takes place where the temperature begins to fall and thus, the metabolic activity decreases.
- Finally, during the last phase, called *curing, maturation or aging phase* which lasts almost a month after the third stage, the compost allows phytotoxic compounds such

as ammonia, which is responsible for high toxicity levels, to be further oxidised. The ammonium ions that still exist in the compost are oxidised to nitrate and consequently, the toxicity is reduced (Barton, 2005; Jenkins, 2005).

1.2.1 Compost production in Donarbon

The leachates used for experimental needs were provided by Donarbon Ltd (Waterbeach, Cambridgeshire). Donarbon processes waste, which comes to approximately 200,000 tonnes per year and also operates a modern operation facility for composting.

For the composting process green waste, kitchen waste, paper, cardboards and kerbside waste are collected. Then, the waste is discharged from the collection vehicles to the waste transfer station (Colour plate 1.1 A) where it is stored for two weeks. A loader follows, to transport the waste to the shredder (Colour plate 1.1 B), which degrades the waste material into smaller pieces. Afterwards, the shredded material is transferred to the composting system consisting of two arrays of clamps and stored there for two days per clamp (Colour plate 1.2 A). The clamps have been constructed on a concrete base and are each able to accept around 180 tonnes of waste. They are sealed and equipped with sensors in order to monitor the oxygen, carbon dioxide and temperature without opening them. Due to aerobic activity of the microorganisms that exist within the waste; there are air pipes inside the clamps providing the necessary air levels. During the respiratory process of the microbes, the temperature rises to approximately 60°C and this temperature is maintained for a minimum of two days. This procedure is repeated after a week when the waste mass is transported to the second clamp. After this period, the maturation phase follows where the waste is transferred to an open-air window. The pre-described process lasts eight weeks in total and complies with the British standards of the British Standard Institution Publicly Available Specification (BSI PAS 100, 2005) for composted materials.

During the clamps phase, leachate is produced and collected. It is not allowed by the European regulations to enter the drainage system due to pesticides and other toxic compounds that it may contain. As a result, it needs separate treatment and so is stored in a tank (Colour plate 1.2 B). This compost leachate was treated using electrochemical peroxidation process (ECP) aiming to make it compatible with the European Regulations and

entering the drainage system without causing any damage to the environment. It also comprises a good example of a toxic waste that would benefit from ECP treatment; hence its use for experiments.

(A)   (B)

Colour plate 1.1 Compost transfer station (A) and the shredder (B).

(A)   (B)
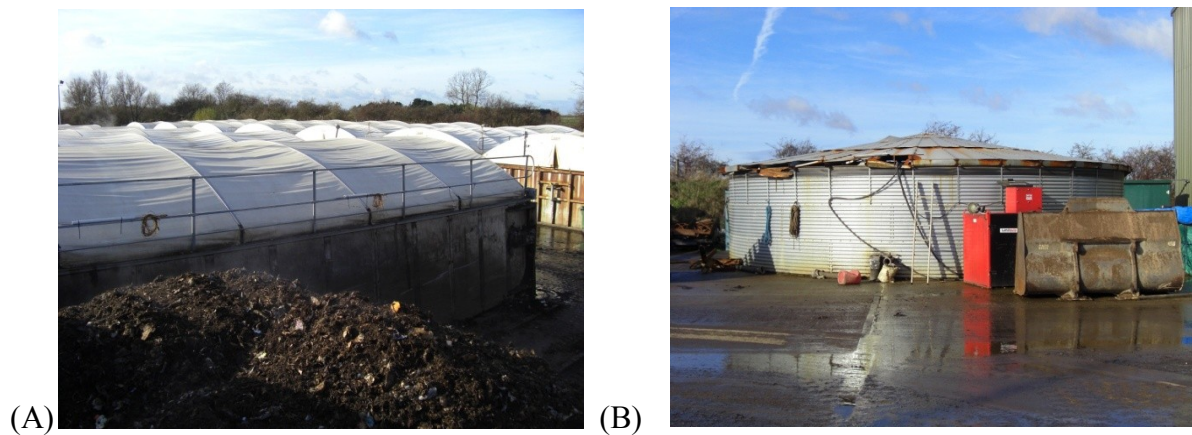
Colour plate 1.2 Compost clamps (A) and leachate storage tank (B).

1.3   Overview of treatment processes

Leachates are composed of a mixture of organic and inorganic pollutants in high concentrations with inorganic salts, ammonia nitrogen, humic acids, xenobiotics and heavy metals, which have to be removed due to their undesirable effects on the environment or their

toxicity. The composition of the leachates is dependent on the climatic conditions, their age, the type and composition of the waste (Renou *et al.,* 2008).

Leachates are divided into the following three categories based on their age:

- ➢ Recent or young leachates, when their age is up to 5 years
- ➢ Intermediate leachates, between 5 and 10 years
- ➢ Old leachates, over 10 years (Renou *et al.,* 2008).

The technological processes that have been used for leachate treatment can be categorized as:

- (i) biological treatments,
- (ii) physico-chemical treatments and
- (iii) combinations between (i) and (ii)

Biological treatments are based on providing the optimum conditions for microorganisms to grow regardless of the type of wastewater. The microorganisms are utilized to convert a mixture of inorganic compounds of K, Mg, Ca, S, P, N and dissolved organic compounds into gases and integrated into cell tissues. Many of the biological processes are comprised of complicated systems, which include microorganisms adjusted to remove particular pollutants.

The main biological techniques that have been applied for treating wastewater are the following:

- a. *Rotating Biological Contactors* is an example of biological filter technology. The advantages of this system include low energy demand, simple operation, low sensitivity to the load of toxins, good settling features of the sludge and resistant to cold. It is usually adjusted to small communities. Rotating Biological Contactors have also got disadvantages including the high capital cost and low performance comparing to other biological treatments (e.g. aerobic activated sludge) (Wiszniowski *et al.,* 2006).

- b. *Aerobic activated sludge* is based on the continuous supply of the reactor that contains microorganisms with oxygen and organic material. Then, the microorganisms convert the organic material, under aerobic conditions, partially into CO_2, H_2O, minerals and partially into new microbial biomass. (Wiszniowski *et al.,* 2006).

c. *Anaerobic systems* involve biological degradation of organic and inorganic matter without molecular oxygen to produce carbon dioxide and methanol. (Wiszniowski *et al.,* 2006).

d. *Biological Nitrogen Removal:* There are three main biological treatments which deal with nitrogen removal from polluted water:

1. *Ammonification* happens when the organic nitrogen is transformed to ammonia. Organic nitrogen is firstly converted to amino acids through hydrolysis. Then, the amino acids are further decomposed to produce ammonium or directly encompassed into biosynthetic pathways to support the bacterial growth.

2. *Nitrification* is a process by which ammonia is converted to nitrate by two groups of bacteria which are operating in sequence. The first group, mainly represented by strains of *Nitrosomonas*, conducts the oxidation of the ammonia to nitrite (Equation 1.1). A further oxidation of the nitrite to nitrate performed mainly by strains of *Nitrobacter* (Equation 1.2).

$$NH_3 + O_2 \xrightarrow{\text{Nitrosomonas}} NO_2^- + 3H^+ + 2e^- \qquad (1.1)$$

$$NO_2^- + H_2O \xrightarrow{\text{Nitrobacter}} NO_3^- + 2H^+ + 2e^- \qquad (1.2)$$
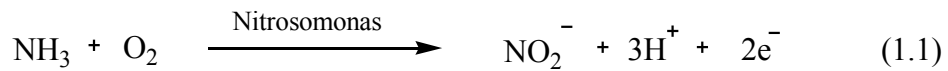
(Wiszniowski *et al.,* 2006).

Denitrification is a process that follows *Nitrification* and transforms the nitrate to nitrogen gas.

$$\text{Nitrate (NO}_3^-) \longrightarrow \text{Nitrite (NO}_2^-) \longrightarrow \text{Nitric oxide (NO)}$$
$$\longrightarrow \text{Nitrous oxide (N}_2\text{O)} \longrightarrow \text{Nitrogen gas (N}_2)$$
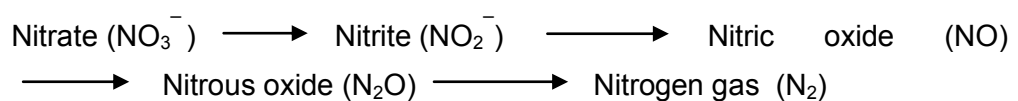
This process is performed from bacteria including *Lactobacillus*, *Pseudomonas*, *Alcaligenes*, *Hyphomicrobium*, *Acinetobacter*, *Thiobacillus* and *Spirillum* (Metcalf & Eddy, 1991). Furthermore, it is more effective in leachates with great amounts of volatile fatty acids (Wiszniowski *et al.,* 2006).

3.   *Anammox* is a process by which the ammonium is converted into nitrogen gas with nitrite used as electron acceptor:

$$NH_4^+ + NO_2^- \longrightarrow 2\ H_2O + N_2 \qquad (1.3)$$
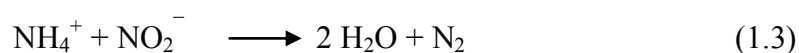
A principal disadvantage of this treatment is the long start-up time. This happens because of the low growth rate of Anammox planctomycetes (Wiszniowski *et al.*, 2006).

Physico-chemical treatments are utilized with biological processes either for better efficacy of the process or for possible application when the oxidation process is hindered by bio-refractory compounds. These treatments are used in order to remove the recalcitrant materials (PCBs, heavy metals) and/or the non-biodegradable ones (fulvic acid, humic acid) from leachates. The physico-chemical techniques that comprise this category are:

(a) coagulation-flocculation,

(b) adsorption,

(c) membrane processes and

(d) chemical oxidation- Advanced Oxidation Processes (AOP).

Finally, due to the stringent regulations for the leachate discharge, the plants are forced to incorporate and combine physico-chemical and biological methods (Wiszniowski *et al.,* 2006).

1.3.1 Advanced Oxidation Processes (AOP)

Advanced Oxidation processes are chemical treatments using different combinations of strong oxidants (such as hydrogen peroxide, ozone or oxygen), catalysts (such as photocatalysts or metal ions) and irradiation (such as ultraviolet or ultrasound) for generating free hydroxyl radicals (HO·) (Table 1.1). AOP have been effective in the destruction of organic and inorganic pollutants that exist in wastewater (Huang *et al.,* 1993).

As presented in Table 1.2, the hydroxyl radical is one of the strongest oxidizers with an oxidation potential of 2.80V and categorized second after Fluorine (E^0 =3.06V). This high potential of HO· radicals enables them to attack the organic compounds and decompose them.

The powerful oxidation capability of HO· is also enforced by its ability to reduce the amount of Chemical Oxygen Demand (COD) of treated wastewater to desirable levels of Maximum Allowable Concentration (MAC) by means of mineralization of the resistant pollutants. In addition, the free radicals enhance the biodegradability of processed effluents for their future biological treatment.

The common characteristic of AOP is the high treatment cost due to high electrical energy resources required for the function of the apparatus comprising of ultraviolet lamps, ozonizers and ultrasound. In contrast, Fenton reaction (H_2O_2/Fe^{2+}) may occur even naturally in several ways such as within biological cells, cloud formation or in the lakes' surface (Prousek, 1996). Consequently, its application to wastewater as a remediation process from the late 1960's was proved successful and cost-efficient.

Table 1.1 Advanced Oxidation Processes

H_2O_2/Fe^{2+} *(Fenton)*

H_2O_2/Fe^{3+} *(Fenton-like)*

H_2O_2/Fe^{2+}/light *(Photo-Fenton)*

H_2O_2/Fe^{3+}-ligand/light *(Modified photo-Fenton)*

H_2O_2/Fe^{2+} (Fe^{3+})/UV *(Photo assisted Fenton)*

H_2O_2/Fe^{3+}- Oxalate

Mn^{2+}/Oxalic acid/Ozone

TiO_2/hv/O_2 *(Photocatalysis)*

O_3/H_2O_2 *(Peroxone)*

O_3/TiO_2/ H_2O_2

O_3/TiO_2/electron-beam irradiation

O_3/UV/ H_2O_2

O_3 /ultrasonics

O_3/UV

O_3 + electron-beam irradiation

H_2O_2/UV

*(Tarr, 2003; Al-Kdasi *et al.,* 2004; Parsons, 2004)

Table 1.2 Electrochemical oxidation potential values for oxidizing agents*

Oxidizing agent	Electrochemical Oxidation Potential (V)
Fluorine	3.06
Hydroxyl radical	2.80
Oxygen (atomic)	2.42
Ozone	2.08
Hydrogen Peroxide	1.78
Hypochlorite	1.4

*(Al-Kdasi *et al.*, 2004)

1.3.2 Fenton's reagent

Fenton's reagent was developed by Henry John Horstman Fenton in 1894. Then, Haber and Weiss (1934) specified the basic mechanism of the Fenton's process and Barb *et al.* (1951) further extended it. Later, Walling (1975) investigated the fate of the produced radical intermediates and the reaction paths of free hydroxyl radicals with organic compounds in the Fenton process (Yoon *et al.*, 2001).

The main advantage of the Fenton system is the complete degradation of contaminants to innoxious compounds such as carbon dioxide (CO_2), oxygen (O_2), water (H_2O) and inorganic salts. The reagent conditions (for example the concentrations of the Fe^{2+}, Fe^{3+} and H_2O_2) and the properties of the treated wastewaters (i.e. temperature, pH, quantity and type of inorganic and organic components) are considered as key characteristics of the Fenton process for treating wastewater (Yoon *et al.,* 2001). Furthermore, during the oxidation procedure there is no formation of chlorinated organic compounds as happens during chlorination or ozonation.

Mechanism of Fenton's reagent

Fenton's reagent is a solution which comprises of hydrogen peroxide and ferrous iron and can be used as an oxidizing process in order to destroy toxic organic pollutants from wastewaters. It produces hydroxyl radicals ($OH\bullet$) according to the following reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$$
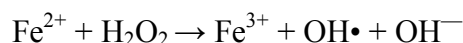
Ferrous iron (Fe^{2+}) initiates the above reaction and also acts as catalyst at the decomposition of hydrogen peroxide (H_2O_2). In order for the hydroxyl radicals to be produced, a complicated reaction sequence is performed in a water solution.

Initiation: $\quad Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^- \qquad\qquad$ (1.1)

Termination: $\quad OH\bullet + Fe^{2+} \rightarrow OH^- + Fe^{3+} \qquad\qquad$ (1.2)
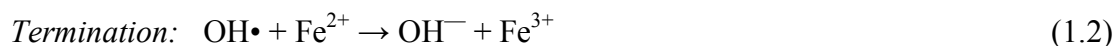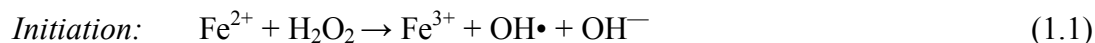
Furthermore, the newly produced ferric ions (Fe^{3+}) can catalyze the hydrogen peroxide, leading to its disintegration into oxygen and water. Additionally, radicals and ferrous ions are formed in accordance with the following reactions (Equations 1.3-1.7):

$$Fe^{3+} + H_2O_2 \rightarrow Fe\ OOH^{2+} + H^+ \tag{1.3}$$

$$Fe\ OOH^{2+} \rightarrow HO_2\bullet + Fe^{2+} \tag{1.4}$$

The above reactions (1.3) and (1.4), in which ferric ions react with hydrogen peroxide, are called Fenton-like reactions (De Laat and Gallard, 1999; Walling and Goosen, 1973).

$$Fe^{2+} + HO_2\bullet \rightarrow Fe^{3+} + HO_2^- \tag{1.5}$$

$$Fe^{3+} + HO_2\bullet \rightarrow Fe^{2+} + O_2 + H^+ \tag{1.6}$$

$$OH\bullet + H_2O_2 \rightarrow H_2O + HO_2\bullet \tag{1.7}$$

In the last reaction, the H_2O_2 captures the hydroxyl radical ($OH\bullet$) and functions as an initiator in the reaction (1.1).

Moreover, organic compounds (RH) can be oxidized by hydroxyl radicals ($OH\bullet$) and organic radicals ($R\bullet$) are produced. Organic radicals are extremely counteractive and can be further oxidized (Lin and Lo, 1997; Venkatadri and Peters, 1993; Walling and Kato, 1971)

$$RH + OH\bullet \rightarrow H_2O + R\bullet \rightarrow \textit{further oxidation} \tag{1.8}$$
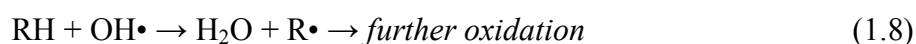
When the concentrations of the reaction agents are not limited, the organic compounds may be entirely degraded by total transformation to carbon dioxide (CO_2) and water (H_2O). In addition, inorganic salts can be produced by substituted organics while the process is continuing.

Furthermore, Walling (1975) has simplified the whole Fenton chemistry by considering the decomposition of hydrogen peroxide to water:

$$2\,Fe^{2+} + H_2O_2 + 2H^+ \rightarrow 2\,Fe^{3+} + 2\,H_2O \qquad (1.9)$$
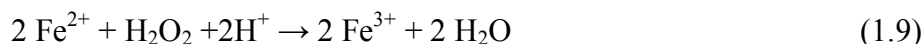
The above reaction proposes that the existence of H^+ is essential in the dissociation of H_2O_2 and also indicates the necessity for an acidic environment in order to produce the maximal quantity of hydroxyl radicals. Arnold (1995) has found that the ideal pH for Fenton oxidation is approximately 3.0. In the case of excess of ferrous ion and low pH, the hydroxyl radicals may be added to the heterocyclic rings or aromatic compounds or even to unsaturated bonds of alkynes or alkenes.

Deduction of a hydrogen atom can be the initiator of a radical chain oxidation (Lipczynska-Kochany *et al.*, 1995; Walling, 1975) as described below:

Chain propagation: $RH + OH\bullet \rightarrow H_2O + R\bullet$ \qquad (1.10)

$$R\bullet + H_2O_2 \rightarrow ROH + OH\bullet \qquad (1.11)$$

$$R\bullet + O_2 \rightarrow ROO\bullet \qquad (1.12)$$
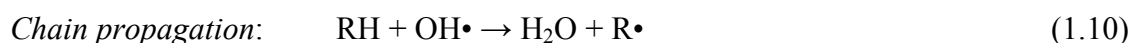
According to the reaction (1.10), organic free radicals are produced which can be subsequently oxidised by Fe^{3+} or reduced by Fe^{2+} or dimerised as showed by the following reactions (Tang and Tassos, 1997).

$$R\bullet + \ Fe^{3+}\text{-reduction} \rightarrow R^+ + Fe^{2+} \qquad (1.13)$$

$$R\bullet + \ Fe^{2+}\text{-oxidation} \rightarrow R^- + Fe^{3+} \qquad (1.14)$$

$$2R\bullet \text{-dimerisation} \rightarrow R\text{-}R \qquad (1.15)$$
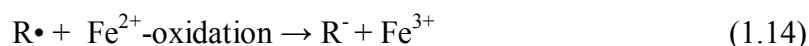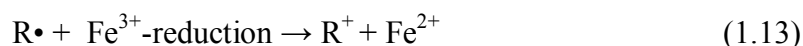
Fenton's reagent chain is constituted by the sequence of the reactions (1.1), (1.2), (1.10) and (1.13) (Neyens *and* Baeyens, 2003). Finally, Fenton's reagent in combination with electrochemistry leads to an Advanced Oxidation Process (AOP) known as electrochemical peroxidation process (ECP) suitable and effective for treating wastewater (Chapter 2).

Important factors affecting Fenton's process

There are various factors that may affect the mechanisms and kinetics of Fenton's process such as the quantity of OH•, the concentration of present contaminants, rate constants and factors related to homogeneity and solubility. For example, if the hydroxyl free radicals are in adequate quantity, the present recalcitrant compounds can be broken down to CO_2 and to some other mineralized products (Tarr, 2003).

The solubility of iron is an important factor in the Fenton oxidation process because the OH• production is dependent on the concentration of Fe^{2+}. At higher pH levels ferric oxides is produced and precipitate. As a result, the OH• formation rate is decreased. Another issue is that if the reduction rate of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) is insufficient, the produced quantity of hydroxyl radicals is often insufficient. At the same time, ferric iron tends to form very stable complexes and its future reduction might be difficult (Tarr, 2003).

1.3.3   Areas of application of Hydrogen Peroxide (H_2O_2)

Since hydrogen peroxide is a powerful oxidant, it has been applied in miscellaneous treatments of organic and inorganic pollutants. Particularly, it has been established in the remediation of wastewater by removing the pollutants such as hypochlorites, sulphites, cyanides, nitrites and chlorine.

Hydrogen peroxide is also used for transforming the gaseous nitrogen oxides and sulphur oxides into the respective acids. Furthermore, it is utilised for the bleaching of the paper and pulp and for organic synthesis. Finally, H_2O_2 has been applied in the industry for the

treatment of surfaces including etching of metals, protecting, decorating and cleaning (Neyens and Baeyens, 2003).

Another aspect of using H_2O_2 is the bio-remediation of biological treatment plants. Hydrogen peroxide decomposition may occur either enzymatically (e.g. catalase) or non-enzymatically (e.g. inorganic catalysts) (Eilbeck and Mattock, 1987). The inorganic catalysts or enzymes naturally exist in the microorganisms. Since oxygen concentrations are limited during *in situ* biodegradation of organic pollutants, injection of H_2O_2 has been successfully attempted (Calabrese and Kostecki, 1989). As a result, biodegradation activity has been enhanced by the dissociation of hydrogen peroxide into water and oxygen (Neyens and Baeyens, 2003). However, the cost of hydrogen peroxide is a limiting factor (Eilbeck and Mattock, 1987).

Low ratios of oxidation, by hydrogen peroxide at reasonable concentrations, may be the reason for the reaction not being effective for great concentrations of inorganic compounds such as cyanides and extremely chlorinated aromatic compounds. The formation of hydroxyl free radicals by H_2O_2 may be produced by ultraviolet (UV) light, ozone and transition metal salts such as iron salt

> H_2O_2 and ultraviolet (UV) light

$$H_2O_2 \, [+ \, UV] \rightarrow 2 \, OH\bullet$$

> H_2O_2 and ozone

$$H_2O_2 + O_3 \rightarrow O_2 + OH\bullet + HO_2\bullet$$
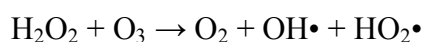
> H_2O_2 and iron salts (Fenton's reagent)

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$$
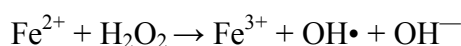
1.4   Toxicity

Since wastewater is a mixture of recalcitrant compounds, the concern of degrading them into smaller compounds, more soluble, less stable but maybe more toxic has been increased. Furthermore, the established regulations have been started showing interest on the toxicity of water entering the drainage system or mixing to surface water.

Subsequently, the wastewater treated or not, causes undesirable effects to microorganisms, plants, animals and biodiversity. The existent toxic compounds can be assimilated to the food chain and the contamination be further passed to humans. Consequently, the increasing environmental awareness has contributed to important technological achievements that can constantly monitoring the aquatic environment.

1.4.1   Toxicity assays

A great variety of toxicity assays have been developed through the last decades. Toxicity bioassay is the measurement of the response of living organisms (microorganisms, invertebrates, fish, plants and algae) when are exposed to toxic substances relevant to a control.

There are many types of toxic compounds that may cause undesirable effects to the living organisms, such as metals, pesticides, radionuclides, organic and inorganic compounds. The types of toxic effects are dependent on the exposure time of the toxicants to the test organisms. They might be *acute,* when the response of the organisms is rapid to a short term exposure ranging from 24 to 96 hours. The toxic effects might also be *subacute, subchronic and chronic*. This division depends on the frequency of the exposure to a chemical. Subacute exposure is when the tested organisms are exposed to a toxicant for one month or less, subchronic for one month to 3 months and chronic for more than three months (Eaton and Klaasen, 2001). However, sometimes the concentrations of the toxicants might be low and not enough to provoke mortality to the tested organisms but sublethal effects on their health. In such cases, sublethal effects may have caused tissue damage which can be estimated

through histologic evidence or creation of tumours or deterioration of the fins (Kendall *et al.,* 2001).

The parameters measured are based on the life cycle (propagation, growth, lethal effects) or on a biological process, which may be inhibited (respiration, bioluminescence, photosynthesis, transformation of sulphur, carbon or nitrogen, enzymes activity, glucose mineralization) using simple methods in the laboratory to electronic devices and biosensors.

Toxicity assays are significant tests that are based on measuring the impact on the metabolic activity of suitable organisms that are exposed to pollutants relevant to a control. These toxic pollutants result in undesirable effects for the living organisms and the life cycle. They may cause lethal effects to fish and to the other aquatic organisms, diseases and disorders in the biological processes such as inhibition of propagation, growth and migration (Tothill and Turner, 1996).

Toxicity tests can provide useful data in assessing risks to aquatic life and to human health from the release of pollutants into water. Due to the fact that it is not possible to determine the toxicity of only one toxic substance in a mixture of effluents, the use of toxicity assays for whole-effluent utilizing aquatic organisms is a preferable solution. This method is cost-effective and direct means of specifying the toxicity of contaminated water. Furthermore, the physical and chemical analyses alone cannot give efficient results for the potential hazards of toxicity compounds on aquatic biota. Biological tests are also available to monitor the contaminants, which cannot be detected by the limited domain of sensors (Tothill and Turner, 1996).

There are many kinds of bioassays that are accessible and can be conducted in the field or laboratory and monitored automatically or manually. The organisms that are involved in such assays include:

1. algae and plants,
2. microorganisms,
3. invertebrates and
4. fish,

For detecting the toxicity of these compounds and their side effects to living organisms, various bioassays and devices have been developed. Some of devices that have been

commercially available and approved by US Environmental Protection Agency - Environmental Technology Verification (EPA-ETV) programme are listed in the Table 1.3.

Table 1.3 Commercially available device and kits approved by EPA-ETV

Tested organism	Device and Kits	Company
Vibrio fischeri-Luminescent bacteria	MicroTox®, Biotox®, AbraTox Kit®	SDI (UK) Aboatox Ltd (Finland) Abraxis (USA)
Daphnia magna	IQ Tox Test®	Aqua Survey Inc. (USA)
Chlorella vulgaris-Algae	LuminoTox *SAPS®* *Test Kit®*	Lab-bell Inc. (Canada)
Uses membranes from chloroplasts	LuminoTox PECS®	Lab-bell Inc. (Canada)
Thamnocephalus platuyurus-invertebrates	RAPIDTOXKIT®	Microbiotests Inc. (Belgium)

Furthermore, recent studies have developed test batteries consisting of assays using various test species in order to assess the toxicity. Nowadays, the evolution of technology expands rapidly. It is known that the most of the systems cannot detect all the toxicants present in a solution with the same sensitivity. Depending on the species applied for each assay the sensitivity varies. Consequently, by combining different technologies and systems which are sensitive to different groups of compounds you end up having a new technology more advanced than the existed and with higher possibilities to detect more if not all the toxic substances existed in the sample.

1.4.2 Plant and algal assays

Bioassays dependent on the features of variant test plants or algae have also been developed. Assays reliant on plant growth responses are possibly sensitive, but demand a great period of

time for growth to occur. From another point of view, they offer some advantages such as a great range of assessment endpoints (biomass weight, germination rate, enzyme activity), quick test activation with particular advantages for the ecotoxic possible evaluation of the solid wastes (Ferrari *et al.,* 1999) and low maintenance cost. Test species such as *Tradescantia micronucleus* for detecting gaseous compounds in wastewater and tap water (Ma *et al.,* 1992; Ruiz *et al.,* 1992), aquatic flowering plants such as *Ranunculus trichophyllus, Ceratophyllum oryzetorum* and *Alisma plantagoaquatica* for detecting the herbicide simazine in water (Ziqing *et al.,* 1994), Chinese cabbage (*Brassica campestris*) and oats (*Avena sativa*) (Farré and Barceló, 2003) have been employed for identifying herbicides in water. An alternative approach is to use chloroplasts extracted from spinach (*Spinacea oleracea*) for also detecting herbicides (atrazine, simazine, metribuzin, cyanazine and diuron) in contaminated water (Piletskaya *et al.,* 1999) (Chapter 3).

The application of algae in bioassays has demonstrated their usefulness in detecting pesticides, herbicides, crude oil substances and metals. Some species that have already been employed as toxicity indicators are *Selensastrum capricornutum*, *Chlorella fusca* and *Dunaliella tertiolecta*. The key theory of algal application as indicators of toxicity is the inhibition of their growth. However, their main drawbacks in their application are the difficulties in culturing and often there is poor reproducibility between serial assays (Farré and Barceló, 2003).

1.4.3 Microbial bioassays

Microbial bioassays are indicator systems, which can provide toxicity assessments of contaminated water in a rapid, cost-effective and easy way. Many studies have been conducted by utilising microorganisms and are categorised by the measuring criterion including:

i.   Enzyme bioassays, which measure the microbial enzymes activity like adenosine triphosphatases, dehydrogenases and other enzymes (Tothill and Turner, 1996).

ii. Bioassays rely on the mortality or growth of the bacteria. These tests are used in testing the wastewater toxicity and the most frequent used microorganisms are

Spirillum voltans and *Pseudomonas fluorescens*.

iii. Glucose mineralization bioassay which based on the inhibitory effect on the rate that labelled carbon dioxide (CO_2) produced by *Escherichia coli* when [U-14C] glucose is present provoked by toxic compounds.

iv. Bioassays measure the inhibition of respiration.

v. Microbial tests which measure the produced luminescence of *Vibrio fischeri* (marine bacterium) by the means of a luminometer (Chapter 4). Toxalert from Merck (Germany) or LUMIStox from Beckman Instruments (USA) or Microtox from SDI (UK) (Farré and Barceló, 2003) are some of the technologies available in the market.

Furthermore, many microorganisms can naturally occur in wastewater such as *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* or in the environment such as *Vibrio fischeri*. Although the growth procedure of the first category of microorganisms is relatively slow, these microorganisms are more representative since they are used as indicators of any change in the constitution of the samples. Regarding the last category of microorganisms, specific properties of their life cycle are measured as indicators of the existence of toxic compounds. In the case of *V.fischeri*, the inhibition of the emitted bioluminescence is monitored. Finally, microbial assays offer an ethical, cheap and relatively easy way to assess the toxicity. A more detailed description regarding the microbial assays will be referred to Chapter 4.

1.4.4 Invertebrate toxicity assays

Invertebrate toxicity assays are also applied in the evaluation of the toxic hazards. The two widely applied in freshwater invertebrate toxicity assays are the *Daphnia magna* and *Ceriodaphnia dubia* survival and reproduction assays (Tothill and Turner, 1996). The test organisms are exposed to toxic compounds under control conditions. After the incubation period of 21 and 7 days respectively, the living daphnias are enumerated. The application of daphnids has many advantages for routine toxicity tests such as parthenogenetic reproduction, short reproductive cycle and high sensitivity to toxins (Tothill and Turner, 1996).

There are also some other toxicity tests utilizing freshwater invertebrates being indicator organisms and are reliant on the growth of amphipods, stoneflies, mayflies or marine species

including oysters or shrimps. *Artemia salina* is one of the marine organisms which have been widely applied for conducting toxicity assays (Chapter 5). However, many assays have been suggested at the level of estimation, with the aim of achieving experimental simplicity, shorter exposure times, easy handling and reproducibility (Farré and Barceló, 2003).

1.4.5 Fish bioassays

Fish bioassays have been applied in toxicity evaluation for decades. It is known that fish display distinct behavioural and physiological responses to quite low levels of contaminants. As a result, fish monitors were developed as indicators of the quality of the water. The assays often rely on fish mortality as they are exposed to the toxic compound for a maximum of 96 hours. The results are recorded as the percent volume, which is lethal to 50% of the fish within the prescribed time (LC_{50}) (Farré and Barceló, 2003).

There are two kinds of acute toxicity assay applied: the static and the flow-through. Choosing the most appropriate type each time depends on the aims of the assay, the available resource, the effluent features and the test organism's prerequisites. Species like fathead minnow and rainbow trout have been used for acute lethality and growth-rate tests. Other fish biotests are reliant on larval growth and survival or on measurements of ATP as a biochemical indicator of energy stress in white fish-muscle tissue (Couture *et al.,* 1989). Recently, many studies have been conducted to substitute the acute toxicity assays to *in vitro* tests, utilizing cell lines of fish (Babich and Borenfreund, 1991) and redox-mediated biosensors encompassing culture cells of fish (Polak *et al.,* 1996).

In general, fish biotests demonstrate good sensitivities and under certain circumstances would allow real-time analysis. On the other hand, they have some disadvantages, such as standardization problems (e.g. optimisation of environmental conditions) and ethical issues. Finally, they consume time and they need special equipment (Farré and Barceló, 2003).

1.4.6 Higher organisms

Mice and human foetuses have also been applied for estimating the toxicity of Uranium (Lambrot *et al.,* 2006). However, special ethics approval was required for the performance of this study. Thus, higher organisms rarely used as test subjects for toxicity trials of this type.

For the purposes of this study, this category of organisms was neither practical nor desirable.

1.4.7 Biosensors

A biosensor is defined as "a compact analytical device incorporating a biological or biologically-derived sensing element either integrated within or intimately associated with physiochemical transducer" (Newman *et al.,* 2001). In recent years, the interest in the development of biosensors for the toxicity estimation has been increasing due to easy handling, adaptability to on-line monitoring, rapid response and the possibilities of mass production.

In whole-cell biosensors, a living microorganism (like a bacterium) can be immobilized. There are several types of bacterial biosensors like those dependent on

a.   monitoring the activity of photosynthesis of *Synechococcus* (cyanobacterium) electrochemically by using redox mediators (Rawson *et al.,* 1989),

b.   measuring the inhibition of conductivity of an agarose coated polymer which contains immobilised *Saccharomyces cerevisiae* (Palmqvist *et al.,* 1994),

c.   changes in the UV absorption BY the microorganism (Baumstark-Khan *et al.,* 1999) and

d.   amperometric biosensors (Evans *et al.,* 1998) using a chemical mediator to convert electrons from the respiratory system of immobilized bacteria to an amperometric carbon electrode with respect to a reference electrode (Farré and Barceló, 2003).

1.5   Aims and objectives

1.5.1 Aim

The aim of this study was to treat highly contaminated wastewater effectively using ECP and produce water with the minimum of toxicity levels assessed by a set of assays in order to enter the conventional wastewater treatment system.

1.5.2 Objectives

The objectives of this study were:

➢ To optimise the working protocol of the ECP for treating wastewater.

➢ To apply toxicity assays based to different organisms (plants, microorganisms and invertebrates) for assessing the toxicity of single contaminants and mixtures.

➢ To develop novel assays for detecting potential toxic aqueous solutions.

➢ To optimise the working protocol for estimating the toxicity of wastewater samples following the ECP process.
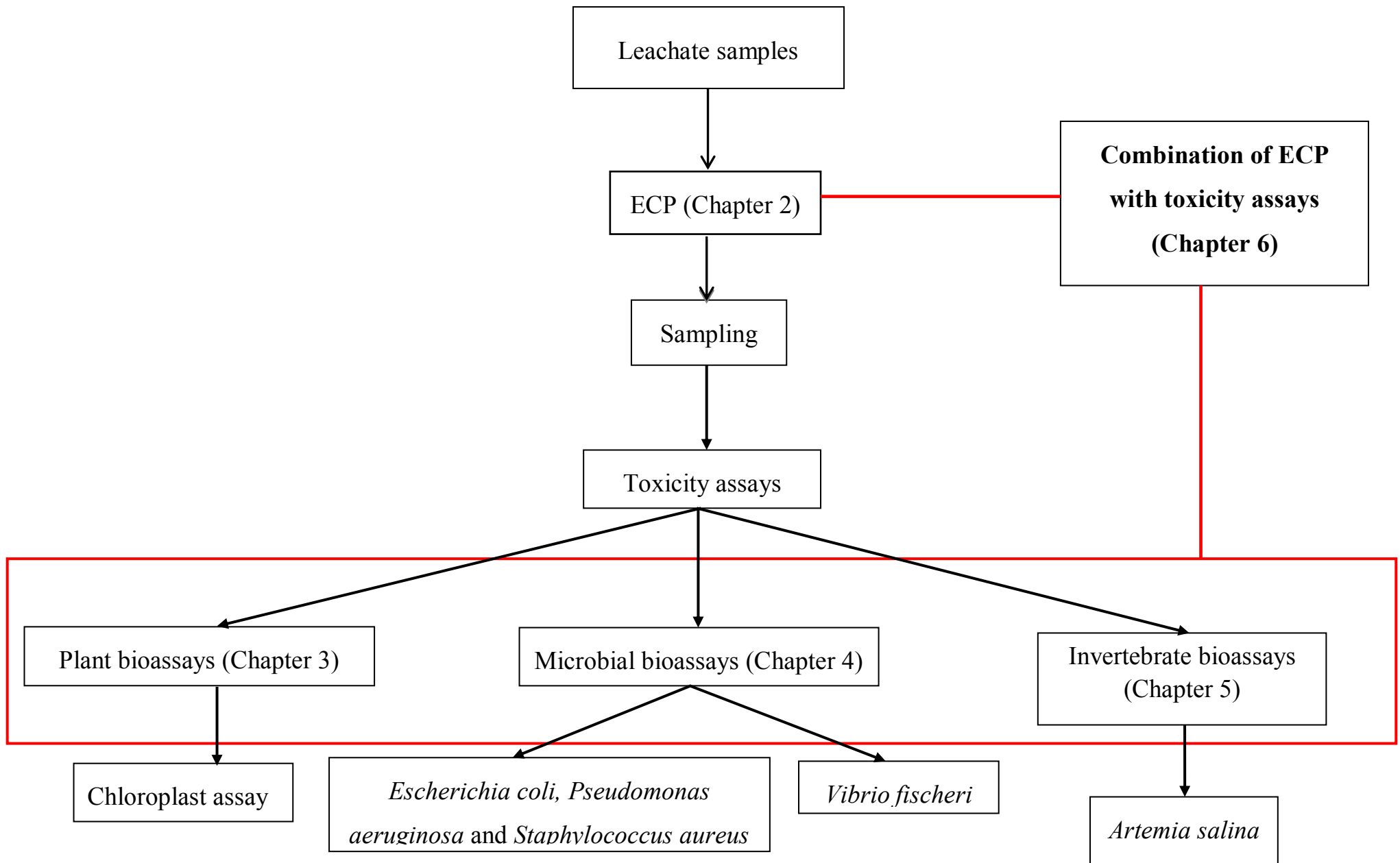
Figure 1.1 Project overview.

Chapter 2 Electrochemical Peroxidation

2.1   Electrochemical peroxidation (ECP)

The ECP is a wastewater remediation treatment, which was firstly investigated by Pratap and Lemley (1994) while trying to develop Fenton's reagent process. Later, they focused on electrochemical peroxidation due to interesting results, which emerged from their previous work (Pratap and Lemley, 1998). Finally, the ECP process was patented from the Centre of Environmental Research, New York's State University at Oswego, USA (Scrudato and Chiarenzelli, 2000). Electrochemical peroxidation is an improved method of producing Fenton's reagent and belongs to the category of Advanced Oxidation Processes. With the conventional Fenton's reagent, ferrous salts (usually sulphate or chloride) are added to water, leading to undesirable effects such as great sludge production and precipitation of ferric salts (Hawksley, 2003). Electrochemistry provides an environmentally friendly method of providing ferrous iron in the solution.

ECP uses sacrificial mild steel electrodes to supply Fe^{2+}. With hydrogen peroxide solution in stoichiometric balance and the application of an electrical voltage, the resultant current produces OH free radicals and, hence, degrades the organic pollutants that exist in wastewater. This reaction occurs, at the anode, according to Equation 2.1:

$$Fe \rightarrow Fe^{2+} + 2e^{-} \tag{2.1}$$

By the addition of hydrogen peroxide, hydroxyl free radicals are produced in a similar way to conventional Fenton's reagent (Equation 2.2).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^{-} \tag{2.2}$$
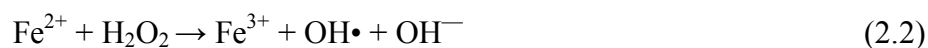
The produced free radicals attack to organic contaminants and degrade them as analytically described in Chapter 1 (section 1.3.2). At the cathode, the produced ferric ions are recycled and reproduce ferrous ions according to the following reduction Equation (2.3):

$$Fe^{3+} + e^- \rightarrow Fe^{2+} \qquad\qquad (2.3)$$

The above reaction is an important and advantageous characteristic of ECP because it permits the iron to behave not only as a reagent but also as a catalyst. This characteristic makes ECP more advantageous than Fenton's process because this recycling supplies the system with ferrous iron continuously and gradually. Consequently, it is less prone to scavenging by free hydroxyl radicals and lower concentrations of reagents such as hydrogen peroxide are needed. Furthermore, the iron salts with chloride or sulphate, usually added during the Fenton treatment, are replaced by iron electrodes. This means that the production of sludge is smaller and there is minimisation of the disposal problem of the ferric salt precipitates (Hawksley, 2003). Finally, removal of heavy metals in sludge is also observed and is further referred to the following section 2.1.2.

2.1.1 Parameters affecting ECP process

According to the US Patent regarding electrochemical peroxidation process, there are some crucial factors that affect the process. Many studies have been conducted in order to optimise these factors and as a result the performance of the process. Some of them have been further investigated and others not to the same degree.

Temperature

According to the above-mentioned patent, heating the hydrogen peroxide or the effluent has similar effects. They report an increase in reaction rate in both cases. The optimum

temperature of hydrogen peroxide should range between 20°C and 70°C and more ideally between 20°C and 50°C. However, experiments conducted by Hawksley (2003) investigating the influence of the temperature varying between 10 and 40°C on the performance of ECP, demonstrated that there were no enhancement of the ECP process performance by increasing the hydrogen peroxide temperature as previously claimed. The reason for this discrepancy is not clear, but it might be due to different H_2O_2 injection methods used in the two studies. Furthermore, the optimum temperature of the effluent ranged between 5°C and 70°C and more ideally between 5°C and 35°C (Scrudato and Chiarenzelli, 2000). Due to the potential cost implications of heating large volumes of water, it is not considered economically viable to raise the effluent temperature, so this was not studied further at this stage. However, it may be scientifically interesting for future work.

pH

Also according to the ECP Patent, the ideal pH values range from 2 to 11 but more ideally between 3 and 5.3. Most of the studies on ECP treatment of leachate samples have been conducted at pH 2.5 - 5 (Pratap and Lemley, 1998; Kurt *et al* 2006; Huang *et al.,* 2008).

Previous studies (Freeman, 2002; Hawksley, 2003; Jury, 2004; Barton, 2005) used the apparatus that was constructed at the Institute of Bioscience and Technology (IBST, Cranfield University, Silsoe). According to these investigations, the optimum pH value was found to be 4.0 for the treatment of landfill leachates, industrial effluents and compost leachates. By lowering the pH of the treated effluent with a strong acid such as H_2SO_4, the conductivity also increased due to the existence of more ions. As a result, the current flow increased and the ferrous generation rate was increased as well. In addition, the solubility of the iron is enhanced at lower pH values, improving its release from the anode and, hence, the performance of the ECP process (Arienzo *et al,* 2001b).

Switching rate and Voltage

The polarity of the electrodes can be switched intermittently in order to improve the reactive area of the electrodes surface and minimise fouling (Scrudato and Chiarenzelli, 2000). In accordance with the Patent, the switching rate and the voltage values were ideally varied

among 0.1-1 cycles/second respectively and 0.5-15V. This could preferably draw an electric current of 0.5 to 10A and current density of 2-50 mA cm^{-2}.

In some former studies on the ECP system used by Freeman (2002), the application of a voltage of 1.9V and a switching rate of 5 cycles/min produced the best results when the aim was the reduction of Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) in treating landfill leachates. By increasing the voltage further, the formation of a precipitate and electrode fouling was increased (Hawksley, 2003). Furthermore, the optimum voltage values are also dependent on the type of leachates and their conductivity (Hawksley, 2003).

Hydrogen peroxide concentration

As reported by Scrudato and Chiarenzelli (2000), the optimal concentration of H_2O_2 should not exceed 100ppm in order to achieve the most effective degradation of the organic pollutants. Freeman (2002) demonstrated that adding lower concentrations of H_2O_2 is at the same degree sufficient. On the other side, Lin and Chang (2000) arrived at the conclusion that by using 750ppm H_2O_2 in treating landfill leachates achieved shorter process time, satisfactory colour removal and COD reduction. Actually, the parameters that must be considered vary each time and dependent mainly on the type of treated effluent.

Concerning the way of adding the desired amount of hydrogen peroxide in the ECP system, it must be preferably added in small doses and in different locations, in order to avoid the high accumulations of H_2O_2 at one place and enhance the system's efficiency. Otherwise, the efficacy of the pollutant oxidation diminishes by producing $HO_2\bullet$, which are scavenged to recycle Fe^{3+} to Fe^{2+} (reactions (1.6) and (1.7), section 1.3.2). This recycling is a slow procedure and results in the consumption of higher amounts of hydrogen peroxide (Pratap and Lemley, 1998; Neyens and Baeyens, 2003).

Electrode plate spacing

Scrudato and Chiarenzelli (2000) suggested that the space between the electrodes should range from 1.3cm to 3.8cm (0.5 to 1.5 in). Other studies have used a 1-1.5cm space between

the electrodes (Arienzo *et al.,* 2001a; 2001b; Lin and Chang, 2000). Freeman (2002) reached the conclusion that the most practicable distance is 1.5cm space between the electrode plates. Actually, little investigation has been performed on the effect of changing the space between the electrode plates, particularly at smaller gaps.

2.1.2 Advantages and disadvantages of ECP

The application of Fenton's reagent for oxidation processes is an effective and well-established method for treating many categories of recalcitrant compounds that exist in wastewater. In many studies, some disadvantages have been mentioned and investigations have been conducted to overcome them.

The main drawback of the Fenton oxidation is the necessity of some salts such as ferrous sulphate ($FeSO_4$), to be added for conducting the process (Brillas and Casado, 2002). However, these may act as pollutants and their removal is necessary before the discharge of the treated effluent. Furthermore, because of the addition of all the reactants at the same time and a great part of the produced hydroxyl radicals are "scavenged" by either the reactants and/or the refractory compounds that exist in wastewater, competitively high concentrations of hydrogen peroxide are required.

Conversely, in the ECP system there is no need for addition of ferrous compounds since the steel electrodes of the system can produce the essential amounts of Fe^{2+} in a constant and controlled rhythm.

Finally, the main advantage of ECP in contrast to Fenton's reagent is the fact of recycling of Fe^{3+} to Fe^{2+}, which means that the iron acts not only as a reactant but also as a catalyst. As a result, the ECP system is provided with iron constantly and progressively. Consequently, the scavenging of the free hydroxyl radicals is much reduced, resulting in a lower H_2O_2 requirement.

On the other side, the ECP process has also some drawbacks such as the formation of solid and soluble hydrous ferric oxides (HFO) resulting in precipitation during the process (Arienzo *et al.,* 2001a). This precipitation incorporates the Fe^{3+} and the biodegraded compounds. It may also contain heavy metals, which may have been absorbed by HFO

(Arienzo *et al.,* 2001a). This by-product should be further analysed before discharge to the environment and the electrodes should be cleaned straight after the treatment in order to prevent their damage.

2.1.3 Electrochemical peroxidation applications

During the past decades an interest on treating different types of contaminated water by electrochemical peroxidation has developed (Table 2.1). Firstly, Pratap and Lemley (1994; 1998) successfully applied electrochemical peroxidation to degrade herbicides such as alaclor, metolachlor, atrazine and picloram. Roe and Lemley (1997) treated insecticides such as melathion and methyl parathion. Huang and co-workers (1999) successfully treated hexamine in petrochemical wastewaters, which constitutes 60% of COD, by electrochemical peroxidation.

Furthermore, Lin and Chang (2000) removed great amounts of recalcitrant organic and inorganic compounds in old landfill leachates. Later, Arienzo *et al* (2001a) used ECP to produce the soluble solid by-product HFO (Hydrous ferric oxides), which could be used as a means of absorbing heavy metals including As, Cd, Be, Cs, Cr, Ni, Li, Se, Pb, Zn and V. Arienzo and co-workers (2001b) also investigated the destruction of polychlorinated biphenyls and the degree of their adsorption on the steel electrodes after ECP treatment. Chiarenzelli *et al* (2001) treated river sediment contaminated by polychlorinated biphenyls with ECP coupled with steam extraction. Panizza and Cerisola (2001) successfully treated industrial wastewater, which contained anthraquinone- and naphthalene-sulphonic acids.

Moreover, Ventura and co-workers (2002) successfully applied the electro-Fenton system in degrading atrazine in acidic medium. Freeman (2002), Quinn (2002), Hawksley (2003), Jury (2004), Barton (2005) and Lydon (2007) treated wastewater through electrochemical peroxidation system and achieved the reduction of colour, deterioration of COD and BOD. Hammami *et al* (2007) obtained, under optimum conditions, full mineralization of DO 61 (direct orange 61), an azo dye, after 6 hours of treatment. Additionally, Huang *et al* (2008) who treated the dye-Reactive Black B (RBB) with different processes, photo-Fenton, electro-Fenton and Fenton reagent, and obtained high percentages of mineralization, particularly by photo-Fenton, and from Özcan *et al* (2008) who treated "the synthetic dye basic blue 3 in

aqueous medium". Finally, Paton *et al.* (2009) applied successfully electrochemical peroxidation process for treating contaminated aqueous leachates and Gutiérrez *et al* (2010) for treating copper smelter wastewater with great concentrations of arsenic and copper.

Consequently, the objectives of the work discussed in this Chapter were:

➢ to improve the ECP performance by utilising the most appropriate H_2O_2 injection method (Run A and B);

➢ to estimate the effect of pH of the samples after the process on the colour removal (Run B) and

➢ to evaluate the effect of low temperatures on the clarity of the samples (Run B).

However, all these experiments were performed in order to optimise the applied protocol when using the ECP process for treating wastewater samples.

Table 2.1 Electrochemical peroxidation applications during the period of 1994-2010.

Authors	Year of publication	Treated Compounds
Pratap, K. and Lemley, A.T.	1994	Herbicide solutions (s-triazines, chloroacetanilides and a picolinic acid derivative)
Roe, R.B. & Lemley, A.T.	1997	Insecticides (melathion and methyl parathion)
Pratap, K. & Lemley, A.T.	1998	Herbicides (atrazine and metolachlor)
Huang, Y-H. *et al*	1999	Petrochemical wastewater (hexamine)
Lin, S.H. & Chang, C.C.	2000	Organic and inorganic recalcitrant compounds in old-aged landfill leachate
Arienzo, M. *et al*	2001a	Metal-contaminated solutions: As, Cd, Be, Cs, Cr, Ni, Li, Se, Pb, Zn, V
Arienzo, M. *et al*	2001b	"Polychlorinated Biphenyl (PCB) destruction"
Chiaranzelli, J. *et al*	2001	River residues contaminated by PCBs
Panizza, M. & Cerisola, G.	2001	Organic pollutants (Naphthalene and Anthraquinone-sulphonic acids)
Huang, Y-H. *et al*	2001	Heavy metals (Pb) and organic compounds (acetate, formate and oxalate)
Ventura, A. *et al*	2002	Atrazine degradation
Arienzo, M. *et al*	2002	Arsenic removal from aqueous solutions
Brillas, E. & Casado, J.	2002	Aniline degradation
Quinn, M.	2002	Landfill leachates

Freeman, B.	2002	Landfill leachates
Hawksley, R	2003	Contaminated water
Jury, S.	2004	Landfill leachates
Barton, P.	2005	Compost leachates
Kurt, U. *et al*	2006	COD in different pH values
Lydon, H.L.	2007	Compost leachates
Giannis, A. *et al*	2007	Olive mill wastewater
Hammami, S. *et al*	2007	Azo dye (direct orange 61- DO 61)
Özcan, A. *et al*	2008	Basic Blue 3 dye (BB3)
Huang, Y-H. *et al*	2008	Dye-Reactive Black B (RBB)
Paton, I.K. et al	2009	Aqueous leachate
Gutiérrez, et al.	2010	Copper smelter wastewater

2.2   Materials and Methods

2.2.1   Reagents

The following reagents were used for all experiments and were of general laboratory grade unless stated otherwise:

Hydrogen peroxide (30%w/w), polyethylene glycol, polyvinyl alcohol, sulphuric acid, 2-propanol and phenol were obtained from Sigma-Aldrich (Gillingham, UK). Sucrose, sodium chloride, dimethylformamide (DMF), hydrochloric acid and copper nitrate [Cu $(NO_3)_2$ $3H_2O$] were provided from Fisher Scientific (Loughborough, UK). Sodium hydroxide was obtained from Acros Organics (Loughborough, UK) and atrazine from Riedel-de Haen, (Hanover, Germany). Finally, Milli Q water was obtained from Millipore (Watford, UK).

Equipment/ Instruments

The following equipment was used:

Palstar DC generator (PS-30M), 3-15V, 0-25 A (Portsmouth, UK), switching unit (Ben Freeman, 2002) and a pond pump, Hozelock Cyprio, (Aylesbury, UK) are basic units of ECP process. A pH-meter, model HI8014, Hanna instruments, (Leighton Buzzard, UK) was used for adjusting the pH values of the effluents. A timer obtained from Fisher Scientific, (Loughborough, UK) was also utilised. The UV-Vis spectrophotometer, CamScan350 and cuvettes obtained from Hellma, (Essex, UK) were applied for measuring the absorbance of the samples. The autoclave obtained from Prestige Medical (Sutton Coldfield, UK) and finally, pipettes of 0.5-10μl and 100-1000μl provided by Fisher Scientific, (Loughborough, UK) were also used.

Effluents

Compost leachate samples obtained by Donarbon Ltd (Waterbridge, Cambridgeshire) were processed. Then, the colour removal was assessed of the untreated samples, of samples taken after frequent time intervals of ECP process and at the end of the process using the UV-Vis spectrophotometer.

ECP apparatus

The electrochemical peroxidation unit consists of a Perspex tank, a power supply, a switching unit, a pump, two tubes with clamps and an effluent reservoir. The Perspex tank has inner dimensions 275 x 150 x 145mm with no lid on the top. This has a capacity of 6.2 l and contains a cassette with two pairs of rods supporting five electrodes each (Colour plate 2.1 A). The electrodes are made from mild steel and their dimensions are 150 x 120 x 1mm. Five of them function as the anode and are attached on the one side of the tank and the other five as cathode on the other side. Plastic spacers of 15mm separate them. Each array of electrodes is connected to a power supply by wires and clips for every electrode plate. This connection method was demonstrated to be preferable by Jury (2004).

The power supply was connected to the switching unit, which was used to change the polarity of electrodes periodically. This was carried out for two reasons: (a) to equalise the corrosion of the electrodes and (b) to prevent the accumulation of waste remnants on electrodes and consequently, preventing the inhibition of electrolysis.

The leachate sample was pumped through the upstream tube and a clamp controlled the flow. The pump (Colour plate 2.1 B) was immersed in the reservoir, which contained a minimum of around one litre of leachate permanently. The whole volume of the leachate was five litres. There is also 75 mm (or 5 spacers) distance between the inlet port and the first electrode for maximizing the contact between electrode and effluent and in order to promote the serpentine leachate flow. The inlet port was located at the highest level of the tank and the outlet port was placed on the opposite side of the tank at its lowest point (Figure 2.1). The outlet port was also connected to a tube with a clamp, which leads to the leachate reservoir. Finally, H_2O_2 was injected either using a syringe between the electrodes (*Method I*) (Colour plate 2.2) or through the 'Y' part connected to the upstream (*Method II*) (Colour plate 2.3).

(A)　　　　　　　　　　　　　　　　　(B)
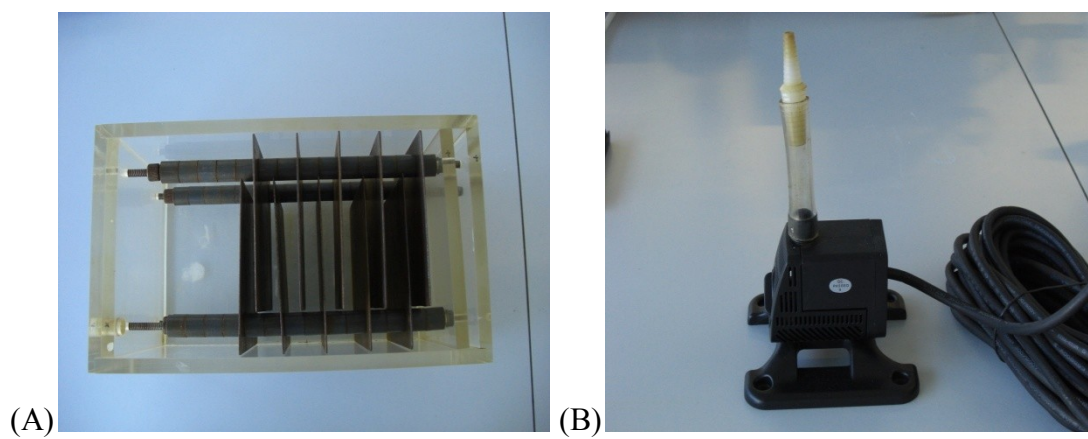
Colour plate 2.1 A top view of the Perspex tank containing the electrodes, the rods and the spacers (A) and the pump (B).
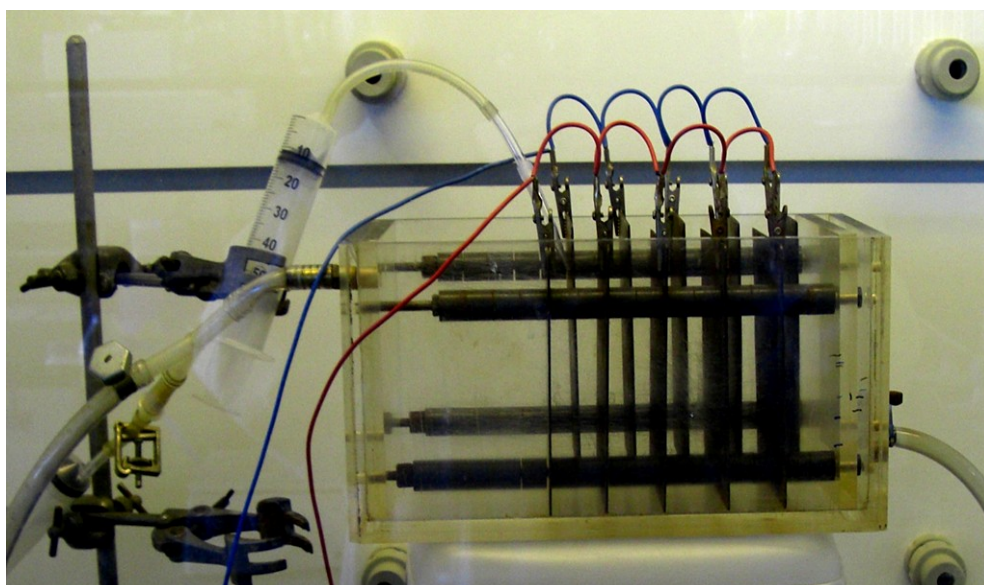
Colour plate 2.2 The H_2O_2 injection method (I) through the syringe between the electrodes.
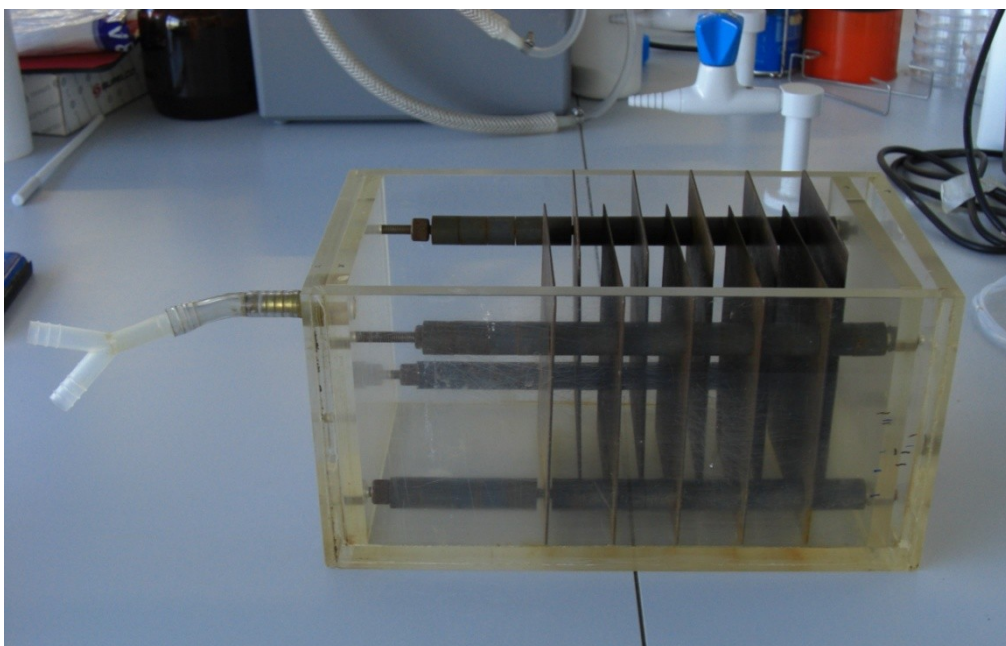
Colour plate 2.3 The H_2O_2 injection method (II) through the 'Y' piece.

ECP experimental procedure

The electrochemical peroxidation system was set up in a fume cupboard due to odour and possible toxic volatiles from leachate samples. Five litres of effluent were poured into the reservoir and the pH adjusted from approximately 7 to 4 using 1M H_2SO_4 or 1M NaOH. Following this, the effluent was pumped through into the tank. The flow rate was adjusted to 1.6 l/min. This was determined using a second reservoir and measuring the collected liquid from the outlet in one minute (Colour plate 2.4).

The DC power supply was set to 3V, electrical current of 0-3A and the switching rate to 5 cycles per minute using the switching unit. Ferrous ions were produced during the pre-treatment step, which lasted for 2 minutes. Then, 25 ml of 2% (v/v) H_2O_2 was added using one of the two injection methods described in the section 2.2.1. The addition of hydrogen peroxide was performed within two minutes (pretreatment step). The treatment time was recorded after this step. The duration of the entire procedure was 40 minutes.

Figure 2.1 An overview of the ECP apparatus.
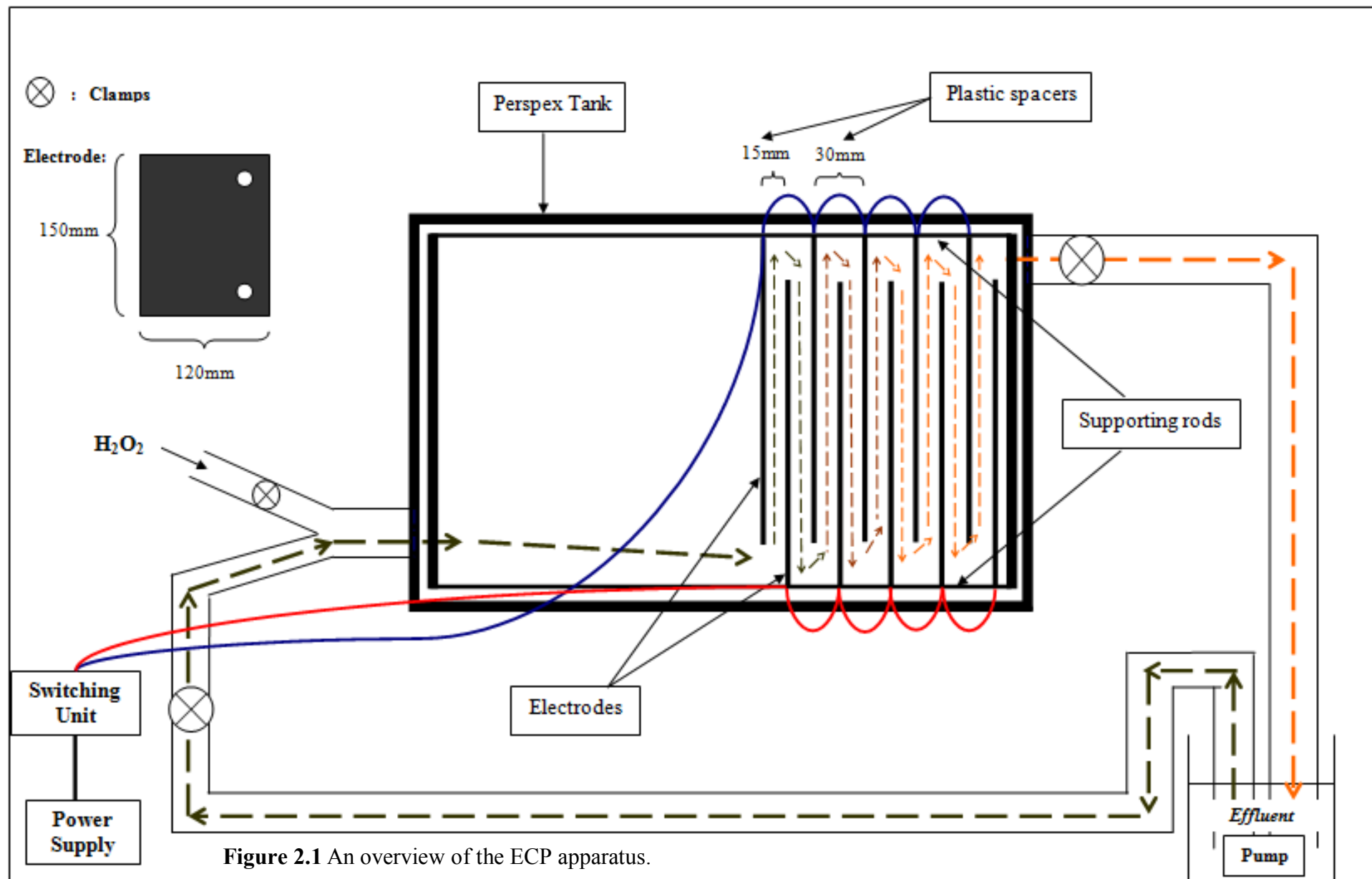
Two processing runs of compost leachate (A and B) were performed as presented in the figures 2.2 and 2.3. Both methods of H_2O_2 injection were applied. Samples were taken from untreated samples and during the treatment at 5, 7.5, 10, 15, 20, 30 and at the 40th minute and stored in 30ml glass bottles. In some cases the analyses were performed immediately and in others the samples were stored in fridge at 4°C for various periods. The analyses followed dealt with colour removal using the UV-Vis spectrophotometer and then the samples were stored in the freezer at -40°C. Finally, their toxicity was estimated using the various assays (Chapter 6).
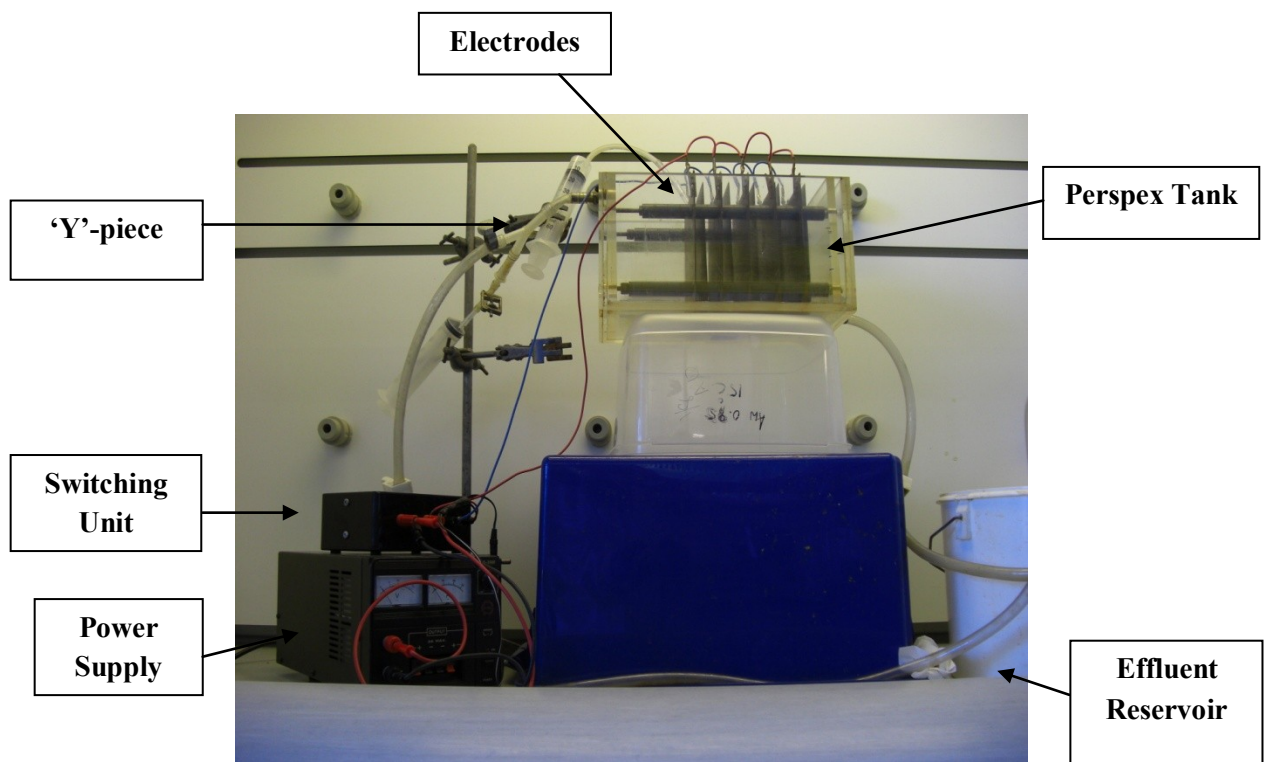
Colour plate 2.4 An overview of the ECP system.
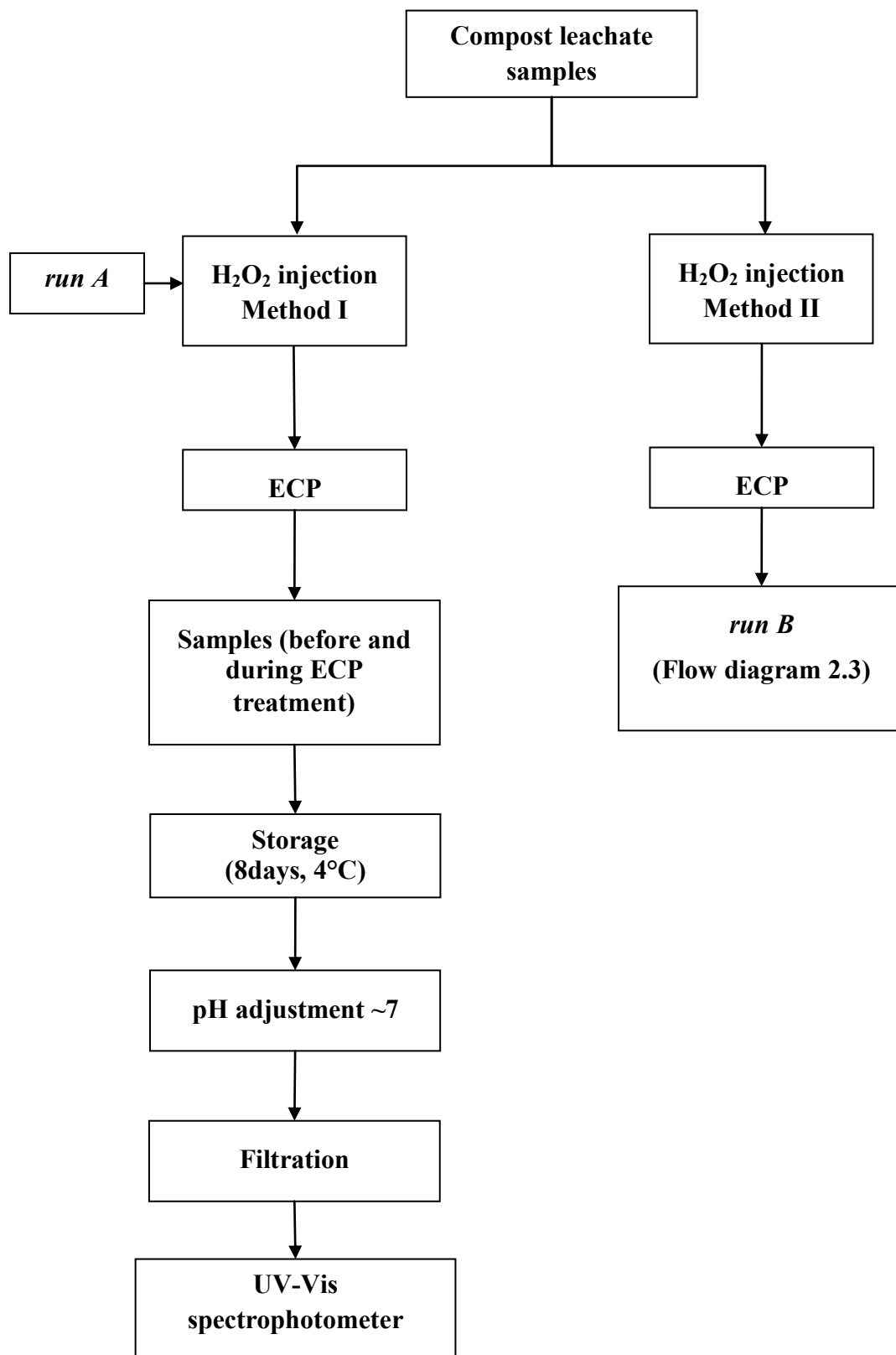
Figure 2.2 Flow diagram of run A experimental procedure.
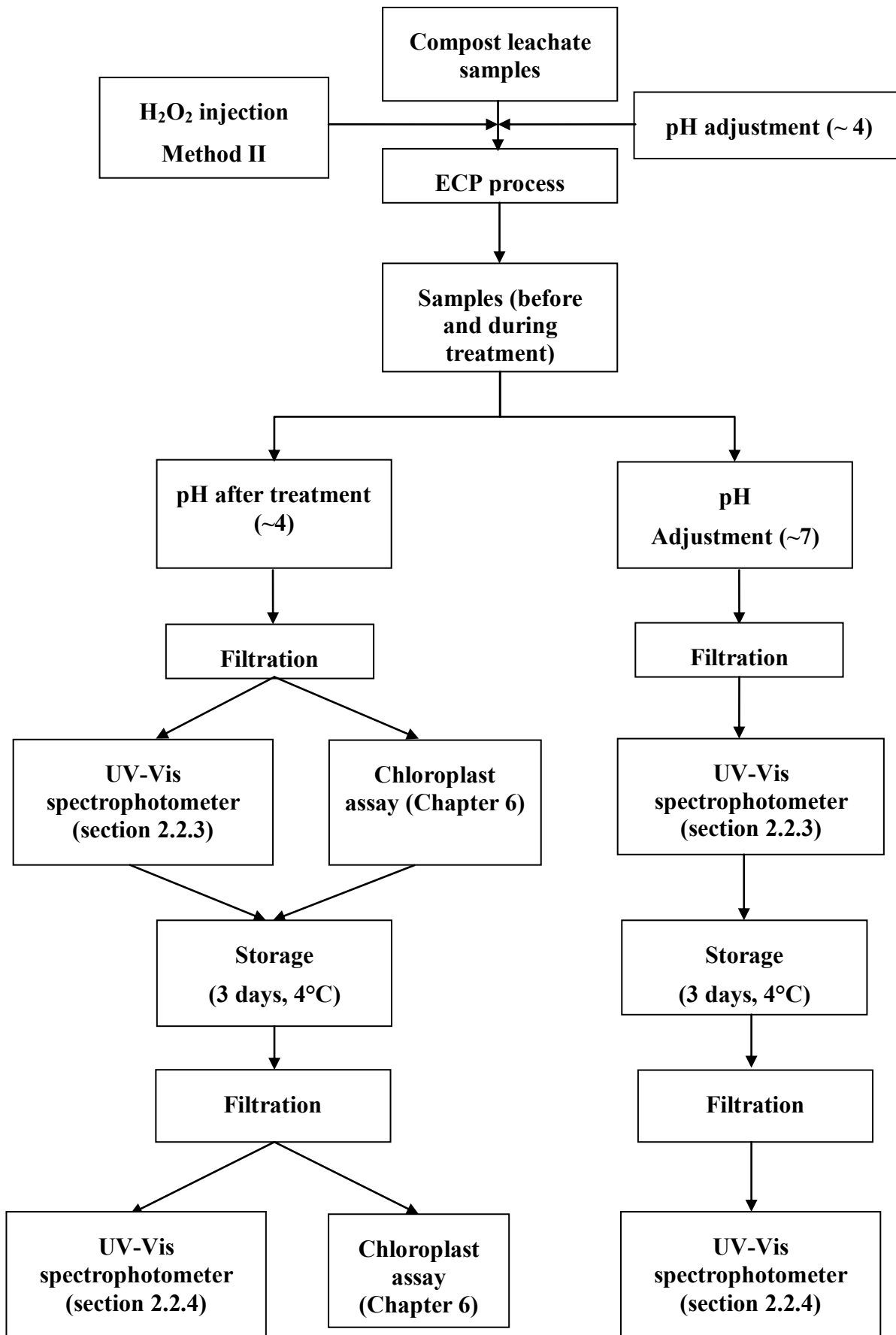
Figure 2.3 Flow diagram of run B experimental procedure.

2.2.2 Study of the effect of H_2O_2 injection method on colour removal

As described in section 2.2.1, 25ml of hydrogen peroxide were injected into the ECP system by two different methods but with the same addition rate (within 2 minutes). The ECP process was conducted under the same conditions of applied voltage, switching rate, leachate pH, flow rate and current density. Samples were taken from run A and B at the same periods of time before the treatment (untreated samples) and after, 5, 7.5, 10, 15, 20, 30 and 40 minutes, as shown in Figures 2.2 and 2.3.

Before the samples scanned between 250-700nm using the UV-Vis spectrophotometer, they were filtered and diluted in distilled water. Many trials of dilutions were performed until adjusting the most appropriate one, which proved to be 20% (v/v) in distilled water.

2.2.3 Study of the effect of pH of compost leachate samples on colour removal

The ECP process was applied as described in section 2.2.1 and under the same conditions. However, the samples were taken in duplicates from run B (Figure 2.3) and tested after 3 days of storage time. Half of these were adjusted to an approximate value pH of 7 (as it was prior to treatment) and the other half were kept at the pH value after ECP treatment (which was approximately 4).

Then, the samples were diluted to 20% in water and scanned between 250-700nm in a UV-Vis spectrophotometer in order to compare the colour removal of the leachate samples with different pH values.

2.2.4 Study of the effect of low temperature storage of compost leachate samples on colour removal

Samples from the experimental run B were tested for their colour removal straight after the ECP process (*first batch*) and after three days of storage in the fridge (4°C) (*second batch*). These samples were collected before the start of the ECP treatment (untreated samples) and after 5, 7.5, 10, 15, 20, 30 and 40 minutes of ECP process. The samples (pH 4) were filtered and diluted (20%) before being scanned between 250-700nm through UV-Vis spectrophotometer.

2.3 Results

2.3.1 Outline of the experiments

Compost leachate samples were treated using ECP and the colour removal was examined

  a. by adjusting two injection methods of hydrogen peroxide,
  b. by re-adjusting the pH to the initial value (approximately 7) and compare it to the pH after the treatment (approximately 4) and
  c. by evaluating the effect of low temperatures on the clarity of the samples.

The results of the aforementioned experiments are displayed in the following sections.

2.3.2 Study of the effect of H_2O_2 injection method on colour removal

The two injection methods were applied during ECP treatments, which performed one week apart. The pH of leachate samples were firstly adjusted to approximately 7 as it was before the treatment for both ways of injection of hydrogen peroxide. Then, they were scanned using UV-Vis spectrophotometer between 250 and 700nm. Deterioration of the optical density (O.D.) was

mainly observed among the samples of the injection *method I* especially within first five minutes of ECP process. The O.D. value for the untreated sample was 1.155 and after 5 minutes of ECP treatment was 0.254. The rest samples did not show any particular change to the absorbance values (Figures 2.4 and 2.5).
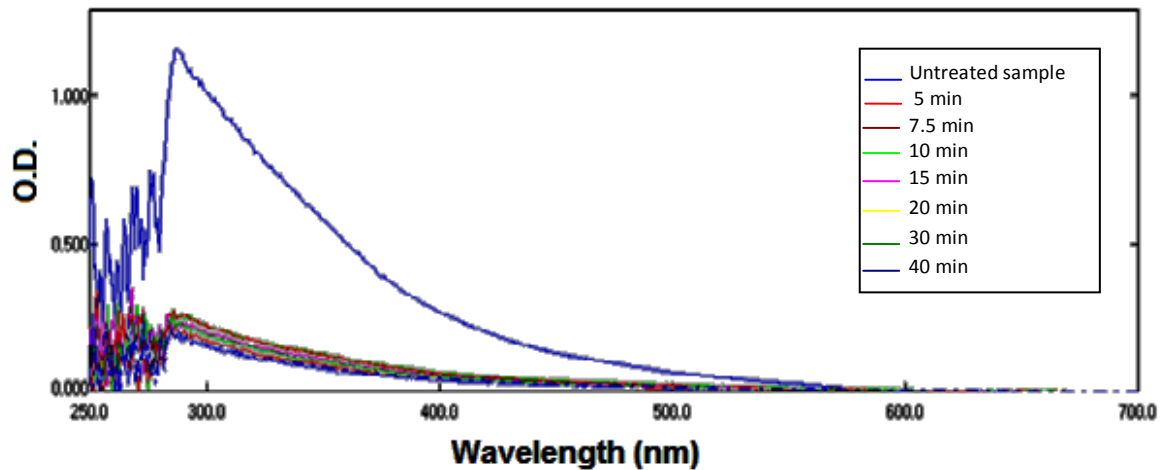
Figure 2.4 Absorbance profiles of untreated samples and after 5, 7.5, 10, 15, 20, 30 and 40 minutes of ECP process using injection method I.
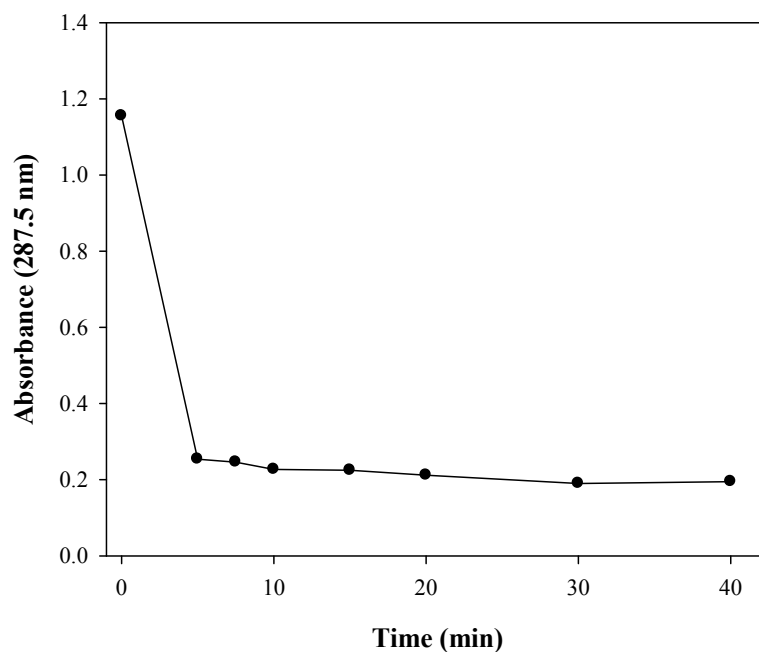
Figure 2.5 Absorbance reduction observed during treatment time (min) at 287.5 nm using injection method I.

By adjusting the injection *method II* and under the same conditions, the absorbance also reduced during the first five minutes of the process but not at the same degree. The untreated sample had an absorbance value of 0.257 which reduced to 0.172 after the first five minutes of the process (Figure 2.6).
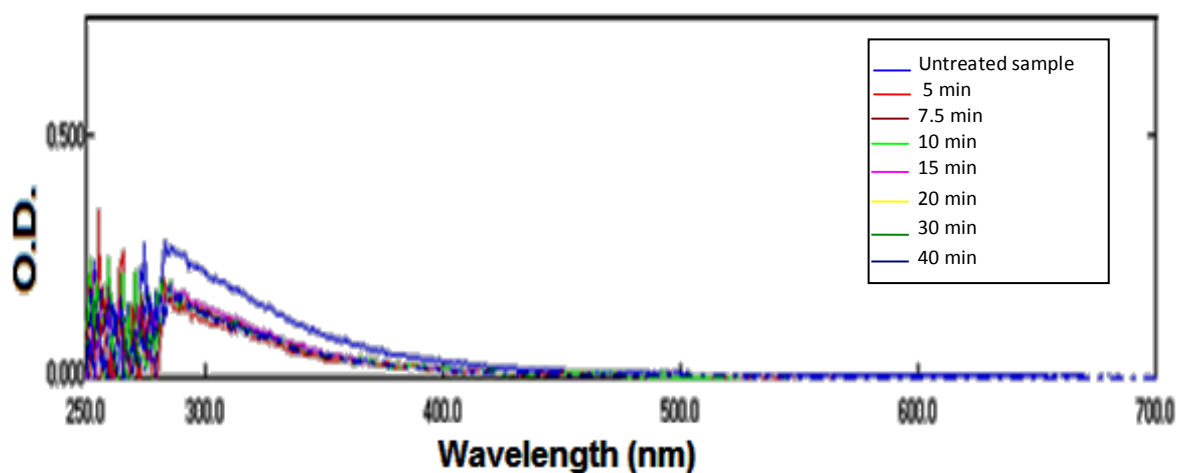
Figure 2.6 Absorbance profiles of untreated samples and after 5, 7.5, 10, 15, 20, 30 and 40 minutes of ECP process using injection method II.

In colour plates 2.5 A and B are illustrated the samples prior to and after the ECP treatment using both H_2O_2 injection methods respectively.

(A)   (B)
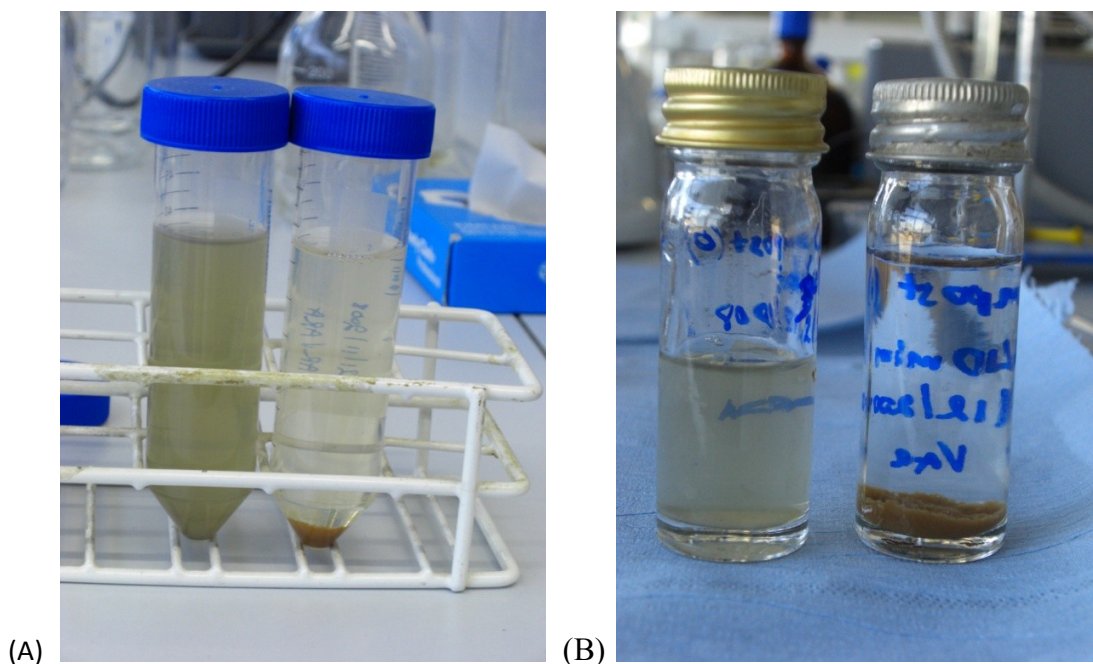
Colour plate 2.5 Samples before and after the ECP treatment using injection method I (A) and II (B).

2.3.3 Study of the effect of pH of compost leachate samples on colour removal

Samples were obtained from run B and after a storage period of 3 days in 4°C were divided into two parts. The pH of the first part was not further processed and was approximately 4. The second part was re-adjusted to ~7 which was the initial pH value of the leachate samples. The samples were then scanned between 250-700nm using a UV-Vis spectrophotometer. The effect of different pH values was monitored (Figures 2.7 and 2.8). The most significant colour change occurred within the first 5 minutes of the process. This was most noticeable on the samples without pH re-adjustment (pH 4), where the absorbance decreased from 0.323 to 0.052 (Figure 2.9).
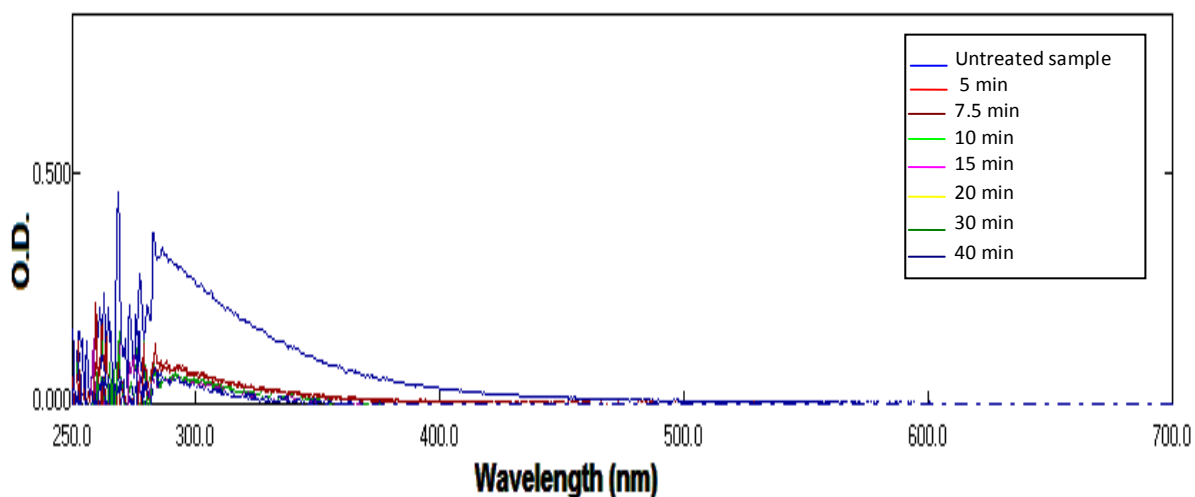
Figure 2.7 Absorbance profiles of untreated samples and after 5, 7.5, 10, 15, 20, 30 and 40 minutes of ECP process of pH 4, using injection method II after 3 days of storage time.
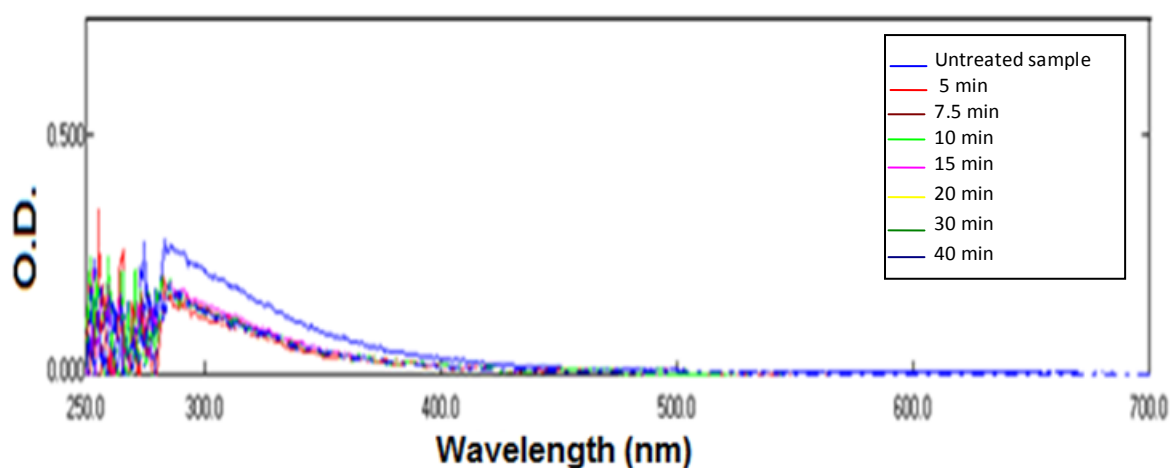
Figure 2.8 Absorbance profiles of untreated samples and after 5, 7.5, 10, 15, 20, 30 and 40 minutes of ECP process of pH 7, using injection method II after 3 days of storage time.
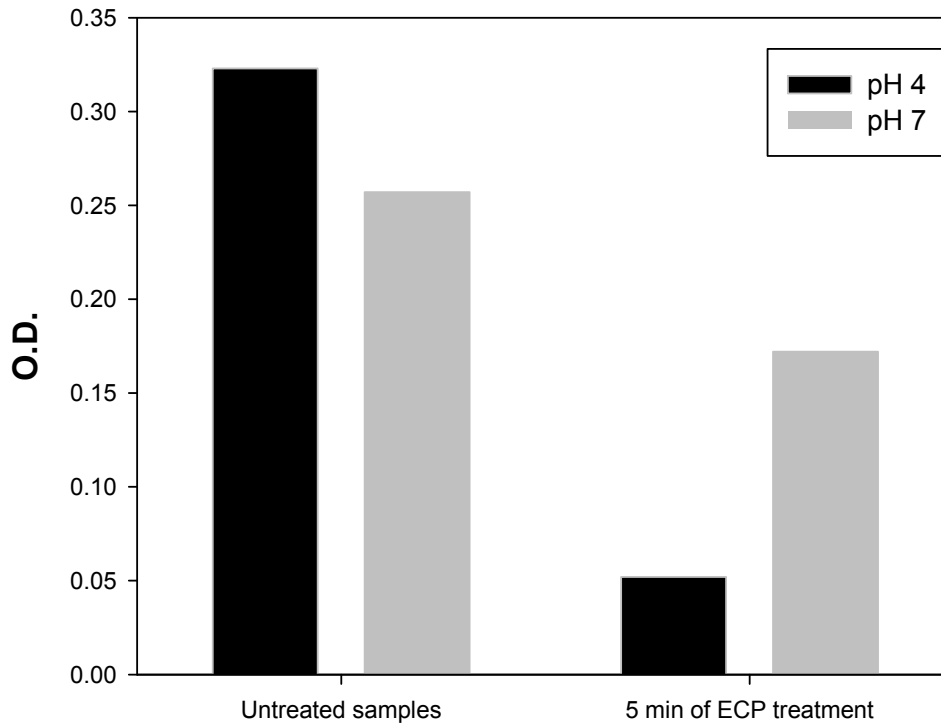
Figure 2.9 Absorbance deterioration measured for the untreated samples and after 5 min the samples of pH ~4 and ~7 after 3 days of storage.

2.3.4 Study of the effect of low temperature storage of compost leachate samples on colour removal

As mentioned in section 2.2.4, samples from the experimental run B were tested for their colour removal immediately after the ECP process (*first batch*) and after three days of storage in the fridge (4°C) (*second batch*). During sample storage, colour change was observed (Figure 2.10 and 2.11). At the *first batch,* the absorbance value of the untreated sample was 0.579 and reduced to 0.164 within the first 5 minutes of the process. At the second batch, the untreated sample had an absorbance value of 0.323 and reduced to 0.052 within the first 5 minutes of ECP-treatment.
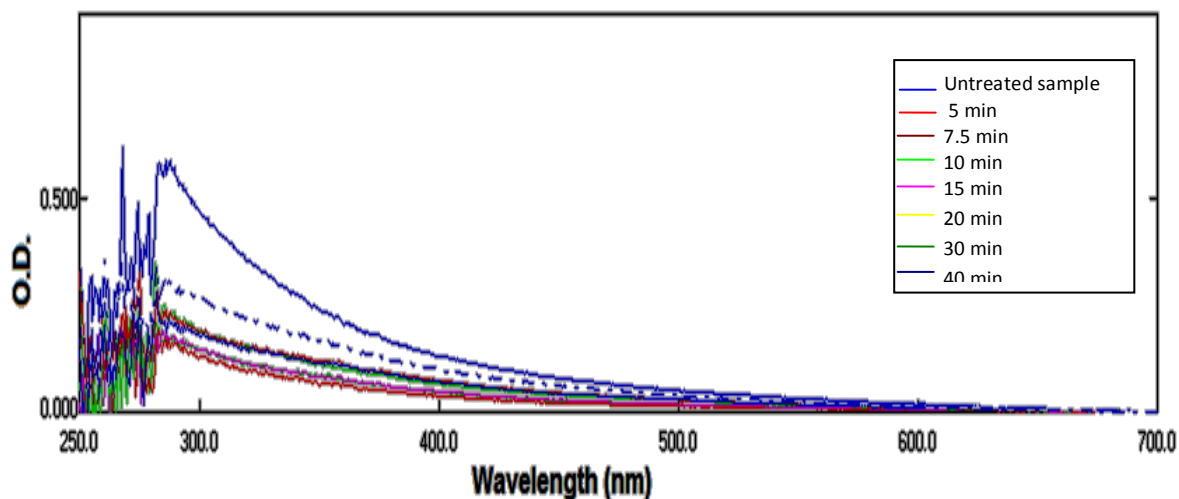
Figure 2.10 Absorbance profiles of untreated samples and after 5, 7.5, 10, 15, 20, 30 and 40 minutes of ECP process.
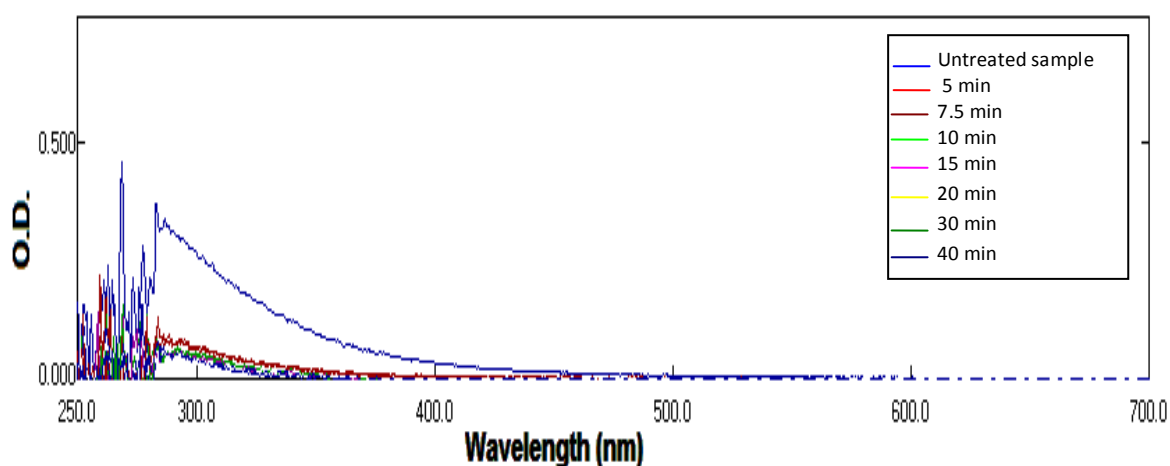
Figure 2.11 Sample Absorbance profiles of untreated samples and after 5, 7.5, 10, 15, 20, 30 and 40 minutes of ECP process after 3 days of storage (fridge, 4°C).

Furthermore, the following colour plates (2.6 and 2.7) display the colour change as a result of storage. The 'before the storage' samples were brown coloured and after the storage period they became colourless. The only exception was observed with the untreated sample which remained grey before and after the storage period.
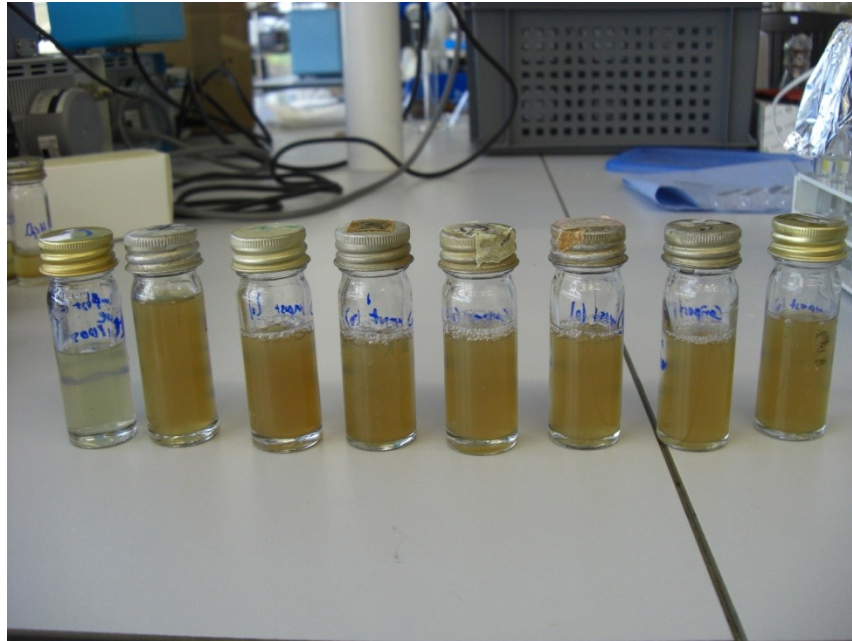
Colour plate 2.6 Untreated sample and samples collected after 5, 7.5, 10, 15, 20, 30 and 40 minutes of ECP process from left to right.

Colour plate 2.7 Untreated sample and samples collected after 5, 7.5, 10, 15, 20, 30 and 40 minutes of ECP process after 3 days of storage (fridge, 4°C) from left to right.

2.4   Discussion

2.4.1   Study of the effect of H$_2$O$_2$ injection method on colour removal

The two experimental runs (A and B) were conducted one week apart in order to study the effect of the H$_2$O$_2$ injection method on colour removal. The untreated samples from both runs had different O.D. values. This may happen due to the settling occurred during the week interceded between the two runs. Furthermore, another reason for this may be the biodegradation occurred within this time by the microorganisms that exist in the effluent.

Additionally, it was observed that the bulk of the reaction occurred within the first five minutes. The absorbance value of the samples of at 7.5, 10, 15, 20, 30 and 40 minutes was similar for both injection methods. However, these experiments were applied for familiarisation reasons and for identifying alternative H$_2$O$_2$ injection methods for optimising the specific process.

2.4.2   Study of the effect of pH of compost leachate samples on colour removal

Since the pH constitutes an important factor affecting the growth of microorganisms and/or invertebrates, the effect of pH of the leachate samples before and after the ECP treatment was examined. Re-adjustment of the pH of the treated leachate to the initial levels (which was approximately 7) showed slight deterioration of the absorbance. This may happen due to the addition of 1M NaOH for re-adjusting the pH and to the formation of turbid solutions. However, better results were achieved by leaving the pH at the same value as it was after the treatment (approximately 4), where the absorption was significantly reduced comparing the untreated sample and the 5-minute-ECP-treated sample. Furthermore, the deduction of the pH of the samples results to the increase of free metal ions in the solutions (Khaled, 2006). This happened due to the competition between metal ions and hydrogen ions for the binding positions on organic and inorganic ligands (Khaled, 2006). However, higher pH values result to the decrease of metal solubility, formation of complexes and their precipitation (Armenante,

1999). According to experimental procedure, the pH was initially adjusted to the second part of samples and then the samples were immediately scanned using the UV-Vis spectrophotometer. As a result, the precipitation of the formed compounds was not fulfilled.

In conclusion, the precipitation of the produced complexes should have been allowed to perform before the scanning of the samples. The expected results would possibly have lower absorbance values than the estimated ones of the samples of pH 4.

2.4.3 Study of the effect of low temperature storage of compost leachate samples on colour removal

Low temperature storage (3 days at 4°C) acted effectively on the colour removal of the ECP treated samples. Enhancement of the particulate settling was occurred and thus the samples became colourless. On the other side, the untreated samples were not affected by low-temperature storage. Subsequently, during the ECP process, degradation of the contaminants was performed which finally resulted to their precipitation. In conclusion, the ECP process coupled with low temperatures are necessary for more environmental friendly water appropriate to enter the drainage system.

2.5   Concusions

The estimation of the effect of some factors including the H_2O_2 injection method, pH and low temperature storage of the samples were investigated in order to be combined with toxicity assays later (Chapter 6).

The H_2O_2 injection methods (I and II) were applied in order to determine if any of them was more effective for contaminants' degradation. The scheduled analyses of the samples were not finally performed because of factors beyond my control.

Additionally, the effect of pH (4 or 7) was analysed. Although the results revealed that pH 4 did not affect the turbidity of the samples, the adjustment to pH 7 increased the turbidity of the

solutions and the O.D. value did not decrease as expected between the tested samples. This fact happened due to the formation of complexes that did not precipitate since the scanning using UV-Vis spectrophotometer conducted immediately after the pH adjustment.

Finally, low temperature storage affected the samples positively as long as the precipitation of the formed compounds performed. More specifically, degradation of large contaminants to smaller compounds occurred during ECP treatment. Then, the precipitation of those compounds was enhanced by low temperatures and clear solutions were obtained. On the other side, the particulate settling did not occur in the untreated samples. However, even though the solutions were not turbid, the solutions could be toxic which will be further assessed in Chapter 6.

Chapter 3 Plant bioassays

3.1   Chloroplast assay

As mentioned in section 1.4.2, bioassays using plants and more specifically spinach (*Spinacea oleracea*) can be applied for assessing the toxicity of aqueous solutions. This assay relies on the binding of several herbicides to the reaction centres of photosynthesis. The employment of the receptor properties of thylakoid membranes appears to be the most straightforward pathway for the bio-recognition test appropriate to detect herbicides which inhibit photosystem II (atrazine, simazine, metribuzin, cyanazine and diuron) (Piletskaya *et al.,* 1999). The key idea of the assay is the catalytic photoreduction of a redox dye by the reaction centres of chloroplasts in photosystem II in relevance with the Hill reaction (section 3.1.2) (Piletskaya *et al.,* 1999).

In particular, chloroplasts are the organelles where the photosynthesis occurs and are approximately 5μm in length (Figure 3.1). The chloroplasts have two membranes, the outer and the inner, and an inter-membrane space between them. The inner membrane encompasses the stroma, where the dark reactions occur. Furthermore, inside stroma, there are membranous flattened sacs called thylakoids. The thylakoid sacs are packed to form granum. Stroma lamellae are the links between the grana. Finally, the thylakoid membranes discrete the stroma space from the thylakoid space (Berg, 2002).
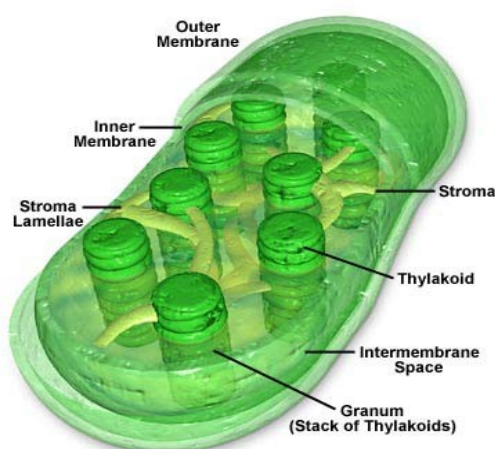
Figure 3.1 Diagram of a chloroplast (Williams, 2008).

3.1.1 Photosynthesis

Among the biological processes occurred in nature, photosynthesis is one of the most fundamental. During photosynthesis, biological systems such as plants, some bacteria and some species of protistans, use light (solar) energy and convert it into chemical energy. The main reaction of photosynthesis is very complicated but it can be summarized by the following simplified schematic equation (3.1):

$$CO_2 + H_2O \xrightarrow{\text{Light}} (CH_2O) + O_2 \qquad (3.1)$$

$$(\text{Berg, 2002})$$
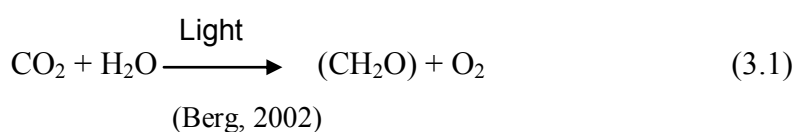
In the above reaction, (CH_2O) represents the carbohydrates produced, such as sucrose and starch. Due to the complicated mechanism of photosynthesis, the involvement of some small molecules and proteins is necessary. In green plants, photosynthesis occurs in chloroplasts where the light energy is trapped by chlorophyll a and b (Figure 3.2). These are pigment molecules which are utilized to produce electrons of high-energy and with high reducing potential. The light reactions are occurring in accordance to "Z-scheme" (Figure 3.3) due to the protein systems through the photosynthetic membrane. The two photosystems (PSI & PSII) interact during the process of photosynthesis. Under typical conditions, the electrons flow firstly throughout photosystem II, then across cytochrome bf and after through photosystem I. Water is the electron donor of the process since 2 molecules of water are oxidized to form a molecule of oxygen for every 4 electrons sent throughout the electron-transport chain. The reduction of $NADP^+$ to NADPH is the final step involving these electrons. NADPH is the flexible agent for driving the biosynthetic procedure. These procedures form a proton gradient throughout the thylakoid membrane that ends to the generation of ATP. Afterwards, the produced NADPH and ATP reduce the CO_2 to 3-phosphoglycerate through the reaction pathway of dark reactions or Calvin cycle (Figure 3.4) (Berg, 2002).
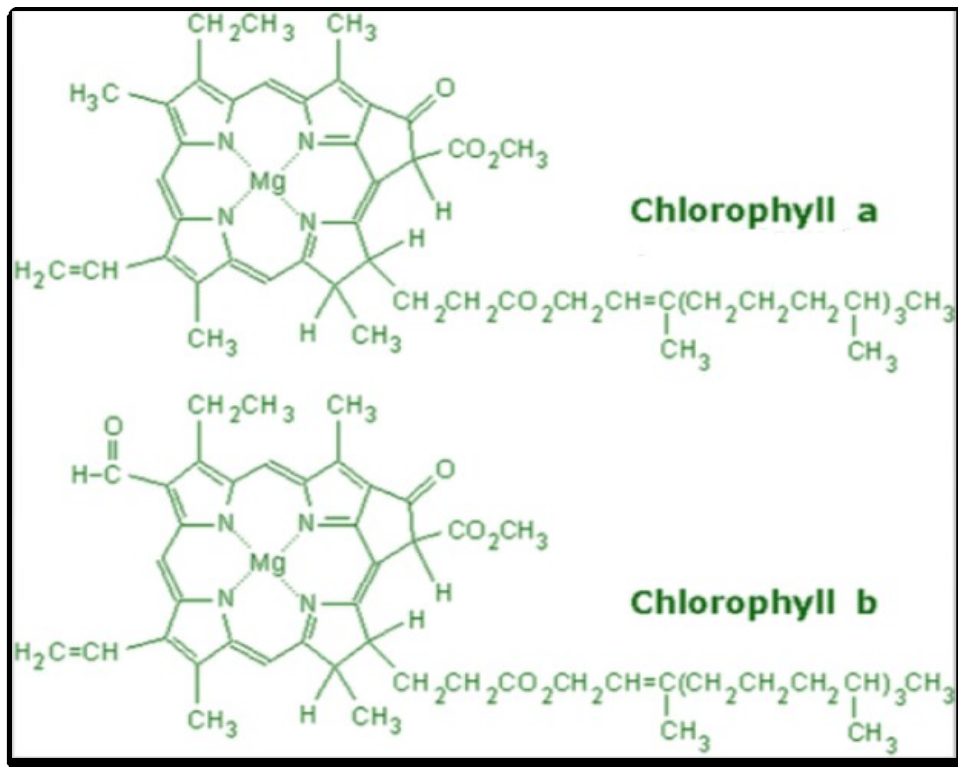
Figure 3.2 Chlorophyll a and b

(Source: http://www.bio.miami.edu/dana/226/226F08_10.html)
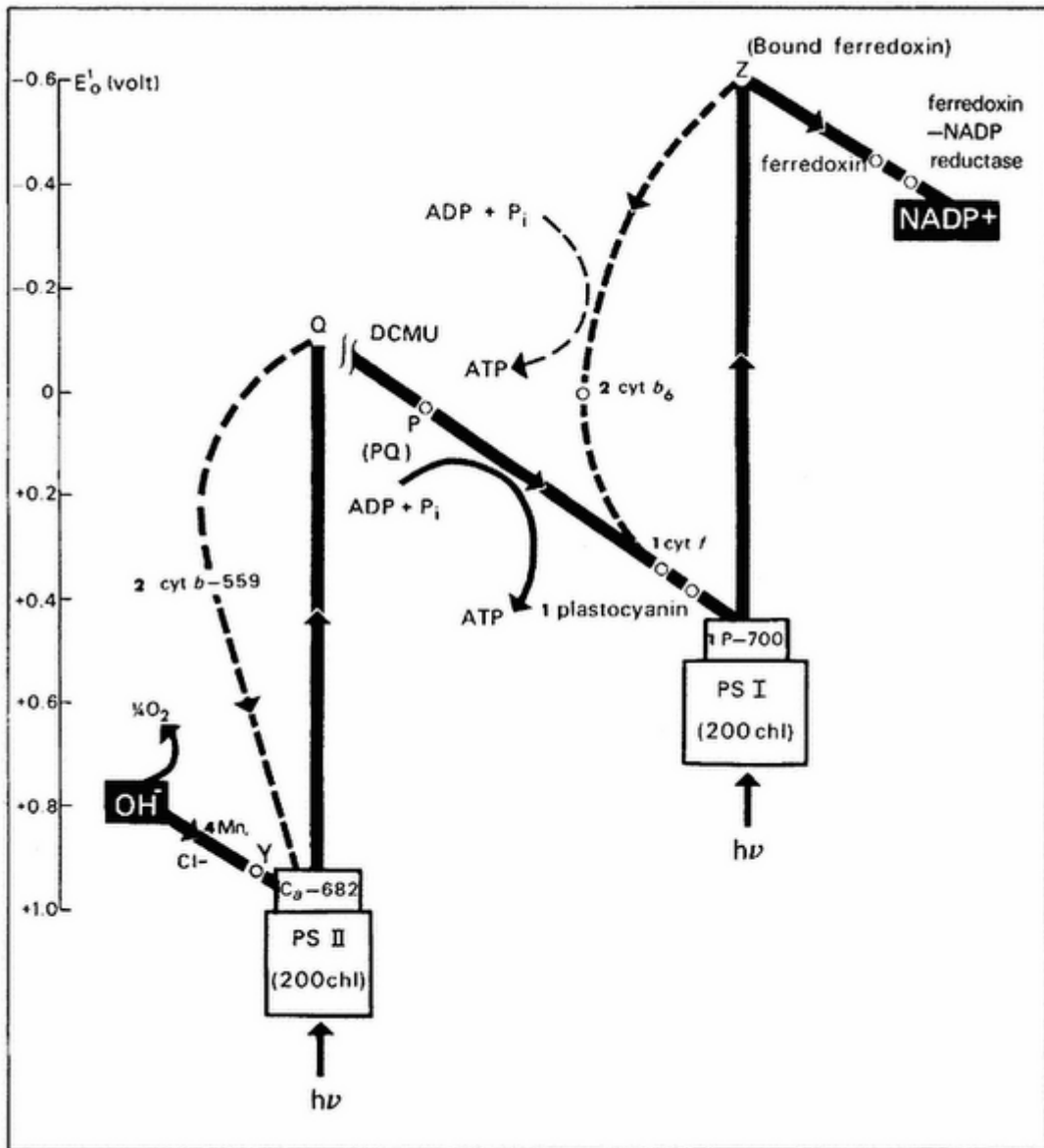
Figure 3.3 The "Z" scheme (Smith, 1978)
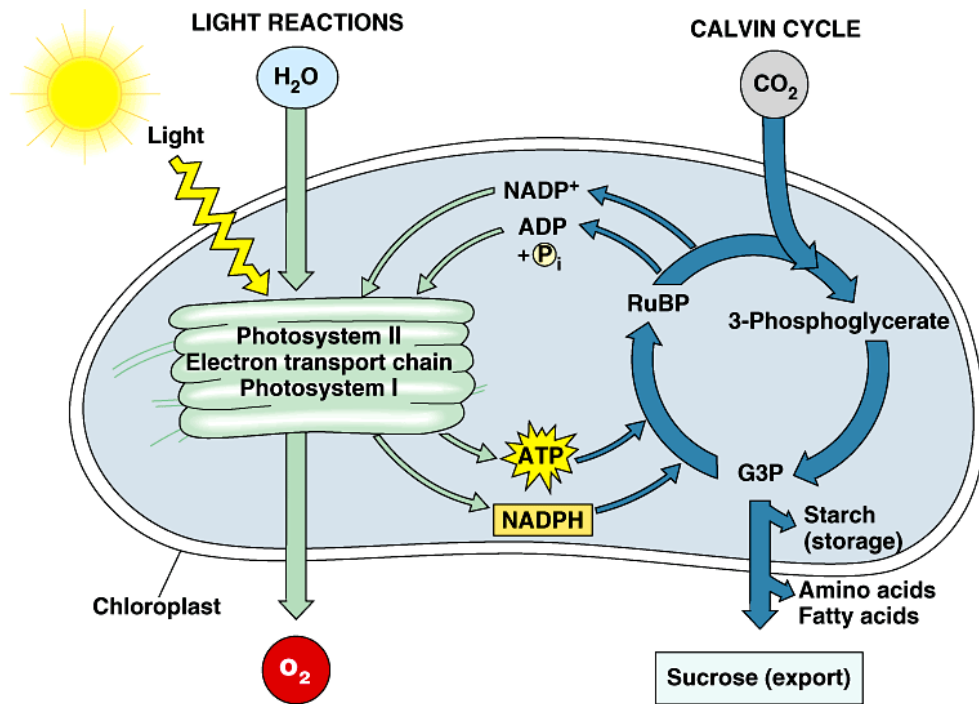
Figure 3.4 Photosynthesis overview

(Source: http://bglarochelle.pbworks.com/w/page/5089496/photosynthesis)

3.1.2 Hill reaction

In 1937, Robert Hill discovered that if isolated chloroplasts are illuminated in the presence of an appropriate electron acceptor, they can generate oxygen even when carbon dioxide is not present. This discovery is a great contribution to the knowledge of the mechanism of photosynthesis, because it reveals that water is the "supplier" of electrons in light reactions. It also confirms that the oxygen derives from water and not from CO_2. Additionally, this discovery showed that experiments can be conducted *in vitro* by using isolated chloroplasts. The Hill reaction is expressed as follows (Equation 3.2):

$$H_2O + A \xrightarrow[\text{chloroplasts}]{\text{light}} AH_2 + \frac{1}{2} O_2 \qquad (3.2)$$
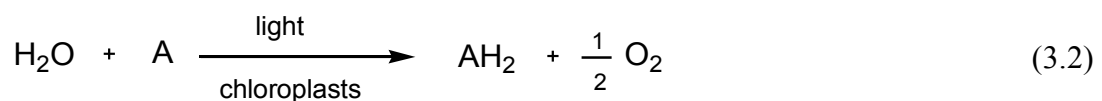
Where A is the electron acceptor which is added to the chloroplast solution and AH_2 is the reduced form. The Hill reaction can only occur in the presence of light. Although during the *in vivo* procedure $NADP^+$ is the electron acceptor, during the *in vitro* could be utilized any artificial electron acceptor.

The most extensively used compound, as electron acceptor, for the Hill reaction is the dye 2,6-dichlophenol-indophenol (DPIP). DPIP accepts the produced electrons from the transport chain of PS II and PS I. This dye is blue in the oxidized form and colorless in the reduced form or green during the assay (chlorophyll colour) (Ventrella, 2007). Subsequently, if the chloroplasts are active and the Hill reaction is performed, a decrease in the absorption of DPIP will be monitored at the range of 550 and 600nm. As a result, the absorbance can be measured and correlated with the toxicity of the compound and its inhibitory effect on the chloroplast functionality.

3.1.3 Tested contaminants

The main tested contaminants were atrazine, copper salts, phenol and sodium hypochlorite and were applied to all toxicity assays of this study (Chapters 3, 4 and 5). All these compounds exist in water (groundwater, rivers, lakes, wastewater, etc.) and they are toxic to the environment, aquatic life, animals and humans.

More specifically, atrazine or [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] is used as an herbicide (Figure 3.5). It is a human made chemical compound and it is believed that there are no natural sources. It lasts for long periods of time in the environment and constitutes a potential contaminant of groundwater and thus its application has been restricted in USA (Arnold *et al.,* 1995). Although atrazine has been banned in Europe, its persistence in soil and groundwater raises the interest in its detection. Regarding its applications, atrazine was mainly used for controlling the weeds in sweet corn and maize. The method of action atrazine involves the inhibition of photosynthesis in susceptible plants. The plants that are tolerant to this herbicide metabolize it. Finally, atrazine has been reported as very toxic for aquatic life and has low bioaccumulation levels in fish.
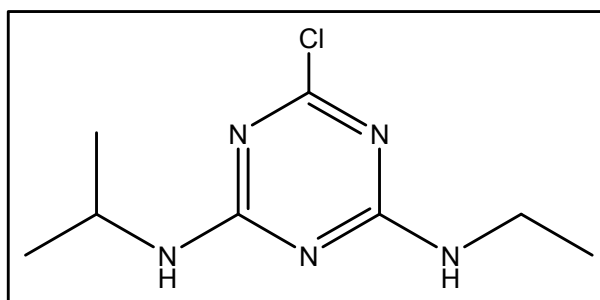
Figure 3.5 Chemical structure of atrazine

Regarding copper, it occurs naturally and is also found in many commercial available products including pesticides, fabrics, leather, ceramics, glass, etc. Copper can be found in sewage treatment because of the drainage systems from homes, roads or from business premises (Dalley, 2001). Furthermore, copper naturally exists in water and food and it is an essential element of the human diet. However, in high concentrations it can damage the habitats of the environment. In addition, copper in its dissolved form is very toxic to aquatic life and especially to early life stages of living organisms (Dalley, 2001). Finally, according to UK government and the Water Quality Objectives that have been established, the allowable concentrations of copper in rivers should be ranged between 0.005mg/l and 0.112mg/l (Dalley, 2001).

Phenol is a volatile organic compound which is mainly manufactured by humans and released to the environment while is applied in industrial processes including the manufacture of disinfectants, plastics and antiseptics. Furthermore, phenolic compounds can also occur naturally from other substance degradation (Daví and Gnudi, 1999). Additionally, high concentrations of phenol can be found in landfill leachate. It is a toxic compound that threatens the aquatic life and generally the ecosystems.

Finally, sodium hypochlorite (NaOCl) is used as a water disinfectant, for water purification, bleaching and odour removal. It is applied for many industrial processes including food, chemical, pharmaceuticals and paper industries. Additionally, sodium hypochlorite is a common used disinfectant of wastewater. Sodium hypochlorite in combination to ammonium salts becomes very toxic and mutagenic for aquatic life. Several studies have been reported that higher total residual chlorine levels than 0.01mg/l can lead to unfavourable effects on freshwater populations of warm water fish and 0.002mg/l on cold water fish (Kelly, 1974; Sawyer, 1976).

To sum up, the objective was to apply a plant based assay for detecting the toxicity of various compounds. A herbicide (atrazine) a heavy metal (copper), an organic compound (phenol), and an inorganic one (sodium hypochlorite) were examined for their toxic effect on spinach chloroplasts. The level of inhibition of the photosynthesis was monitored by evaluating the change in the colour of an indicator compound using a spectrophotometric method.

3.2   Materials and Methods

3.2.1   Reagents

The following reagents were used for all experiments and were of general laboratory grade unless stated otherwise:

Bovine Serum Albumin (BSA), 2,6-dichlorophenolindophenol (DPIP), 4-morpholinepropanesulfonic acid (MOPS) and phenol were obtained from Sigma-Aldrich (Gillingham, UK). Sucrose, sodium chloride, acetone and copper nitrate [Cu $(NO_3)_2$ x 3H2O] were provided from Fisher Scientific (Loughborough, UK). Tris (hydroxylmethyl)aminomethane was provided from Fluka, (Gillingham, UK) and atrazine from Riedel-de Haen, (Hanover, Germany). The sodium hypochlorite used was from the commercially available bleach (Domestos, Uniliever, UK). Finally, Milli Q water was obtained from Millipore (Watford, UK).

Equipment/ Instruments

The following equipment was used:

The centrifuge, ALC International Srl, model: PK131R, (Cologno Monzese, Italy) and a blender obtained from Tesco (Flitwick, UK) were used during the extraction of chloroplasts. The microplate reader, DYNEX Revelation 4.21 and microtitre plates obtained from Sigma,

(Gillingham, UK) were utilised during the assay. The UV-Vis spectrophotometer CamScan350 was applied for measuring the absorbance of the samples. Pipettes of 0.5-10μL and 100-1000μL provided by Fisher Scientific, (Loughborough, UK) were also applied.

3.2.2 Chloroplast extraction

The procedure followed for extracting the chloroplasts from spinach leaves was performed according to Piletskaya *et al.* (1999). Spinach leaves (100g) were kept overnight in the fridge (4°C). Two extraction buffers were prepared on the previous day and also kept in the fridge:

a. Extraction buffer (pH 8)   (1)

 - 0.35M Sucrose

 - 50mM Tris-HCl, pH 8

 - 10mM NaCl

b. Extraction buffer with 1% BSA   (2)

The spinach leaves were washed with tap water and dried on filter paper. Then, they were homogenised in 300ml of extraction buffer (1) using a blender. The homogenate was initially filtered through three layers of cotton wool and then through four layers of cheesecloth. The whole procedure was carried out in ice in order to avoid stressing the chloroplasts.

Then, the filtered liquid was centrifuged for fifteen minutes at 3500 rpm and the temperature kept at 4°C. The resultant pellet (Colour plate 3.1) was re-suspended with the Extraction Buffer (2) containing 1% BSA which aids chloroplast stabilization. Finally, 500μl aliquots in Eppendorf tubes were prepared and stored in the freezer at -80°C.
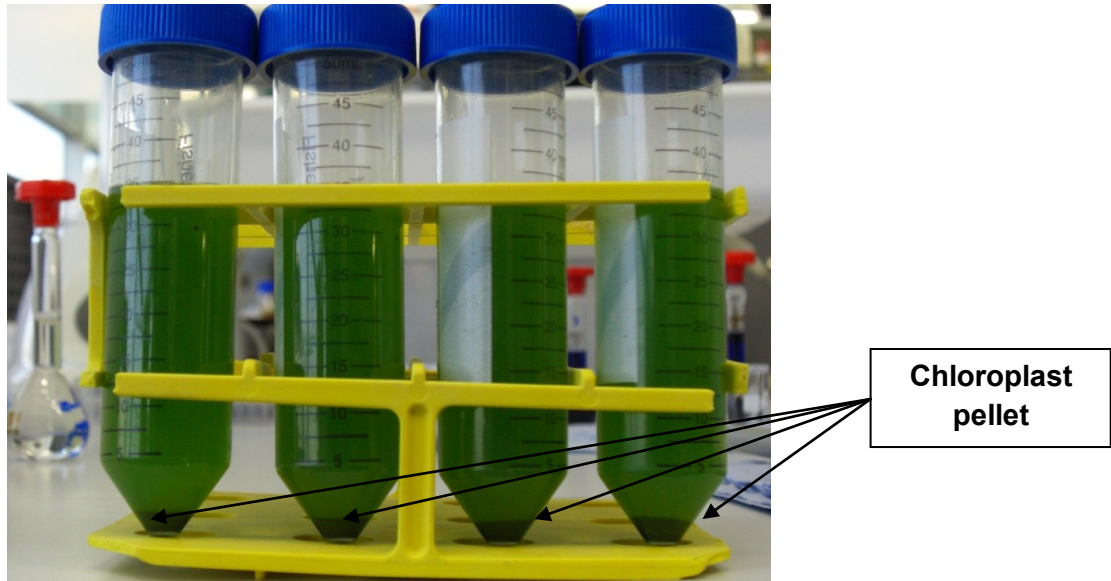
Colour plate 3.1 The solutions after centrifugation when the chloroplast pellets were formed.

3.2.3 Chlorophyll determination

Due to high chloroplast suspension, chlorophyll concentration was determined by dilution of 3μL of chloroplast extract in 1ml of acetone-water (80:20, v/v). Then, the solution was scanned between 550-800nm using UV-Vis spectrophotometer. The concentration was estimated according to Arnon's spectrophotometric method (Equation 3.3) (Arnon, 1949).

[Chl](mg/ml) = [(A_{663} - A_{750}) * 8.02 + (A_{645} - A_{750}) * 20.2] * dilution factor / 1000   (3.3)

Dilution factor = (Volume of the flask) / (Volume of the pipette)

It is also possible to estimate the ratio of chlorophyll a and b concentrations by using the following equation (3.4):

Chla/ Chlb = (2.69 – 12.7 * K) / (4.67 * K – 22.9)   (3.4)

K = (A_{663} - A_{750}) / (A_{645} - A_{750})

A_{645} = Absorbance at 645nm

A_{663} = Absorbance at 663nm

A_{750} = Absorbance at 750nm

3.2.4 Experimental procedure of the chloroplast assay

The extract of chloroplasts was diluted (1:20) in extraction buffer (1). In a 96 well microtitre plate the following were inserted:

    i.   30μl of the pre-described solution;

    ii.   40μl of either distilled water (control) or sample;

    iii.   100 μl of 0.6mM DPIP in 50mM Tris-HCl buffer (pH 8).

Then, the plates were illuminated for either 5 or 10 minutes by a light box consisting of a standard (white lamp) 100W lamp and the absorbance was monitored at 550nm using a microplate reader. Each sample was repeated in triplicate and the mean value was further processed.

3.2.5 Study of the effect of atrazine, copper ions, phenol and sodium hypochlorite on chloroplast assay

The toxicity of atrazine (herbicide), copper salts (heavy metal), phenol (organic compound) and sodium hypochlorite (inorganic compound) was evaluated by applying the chloroplast assay. Atrazine solution (10^{-4}M) was made by mixing 2g of atrazine with 1ml DMF. This mixture was diluted into 99ml Milli Q water. In addition, stock solutions of Cu^{2+} (10^{-2}M), phenol (10^{-2}M) and sodium hypochlorite (10^{-2}) were prepared. The desired geometrically spaced concentrations were performed (Table 3.1) and the methodology described in section 3.2.4 was followed. The samples were tested under the same conditions of pH (8) and dilutions, without being filtered and after 5 and 10 minutes of illumination.

Table 3.1 Preliminary tests for chloroplast assay using atrazine, Cu^{2+}, phenol, and sodium hypochlorite.

Compounds	Concentration (M or mg/l)	
Atrazine	$10^{-4} - 10^{-8}$ or	$21.6\text{-}2.2 \times 10^{-3}$
Cu^{2+}	$10^{-4} - 10^{-8}$ or	$24.2\text{-}2.4 \times 10^{-3}$
Phenol	$10^{-4} - 10^{-8}$ or	$9.4\text{-}9.4 \times 10^{-4}$
Sodium hypochlorite*	$10^{-4} - 10^{-8}$	

*measured as dilution, not absolute concentration

3.3   Results

3.3.1   Results outline

The chloroplast assay was conducted for several compounds including atrazine, copper ions, phenol and sodium hypochlorite in order to assess their toxicity. Atrazine inhibited the Hill reaction and the absorbance values were deteriorated at most concentrated samples. The other compounds were not as toxic to chloroplasts as atrazine since the O.D. values remained almost at the same level as controls.

$$H_2O + DPIP_{ox} \xrightarrow[chloroplasts]{hv} DPIP_{red} + \frac{1}{2} O_2 \qquad (3.5)$$
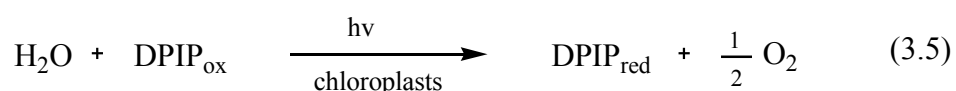
More specifically, when the wells were prepared and before the illumination, the colour of the solutions was dark blue. In colour plate 3.2 is displayed a microtitre plate with one of the tested compounds examined with the chloroplast assay. In particular, after the illumination period, where the Hill reaction was not inhibited as happened in the first (controls) and two last rows of samples (very diluted samples), the colour of DPIP turned to colourless and the green colour of chlorophyll prevailed. In the opposite case, where Hill reaction was inhibited, the colour of the mixture remained dark blue. All the occurred changes in the colour of the solution were evaluated with high accuracy using the microtitre plate reader at 550nm.
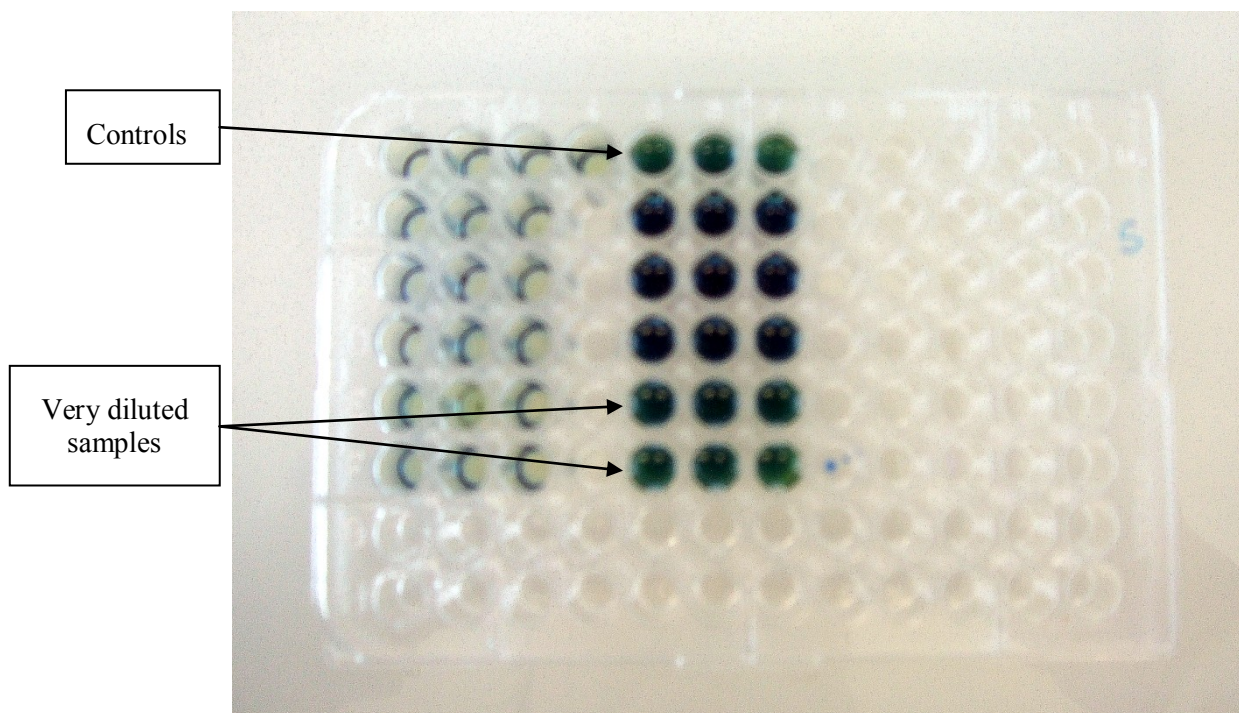
Colour plate 3.2 Microtitre plate with the experimented solution of DPIP buffer, chloroplast solution and toxic compound or distilled water (control)

3.3.2 Study of the effect of atrazine, copper ions, phenol and sodium hypochlorite on chloroplast assay

The chloroplast assay was applied to atrazine, copper salts, phenol and sodium hypochlorite. The chlorophyll concentration was 0.67 mg/ml and its spectrum is displayed on Figure 3.6.
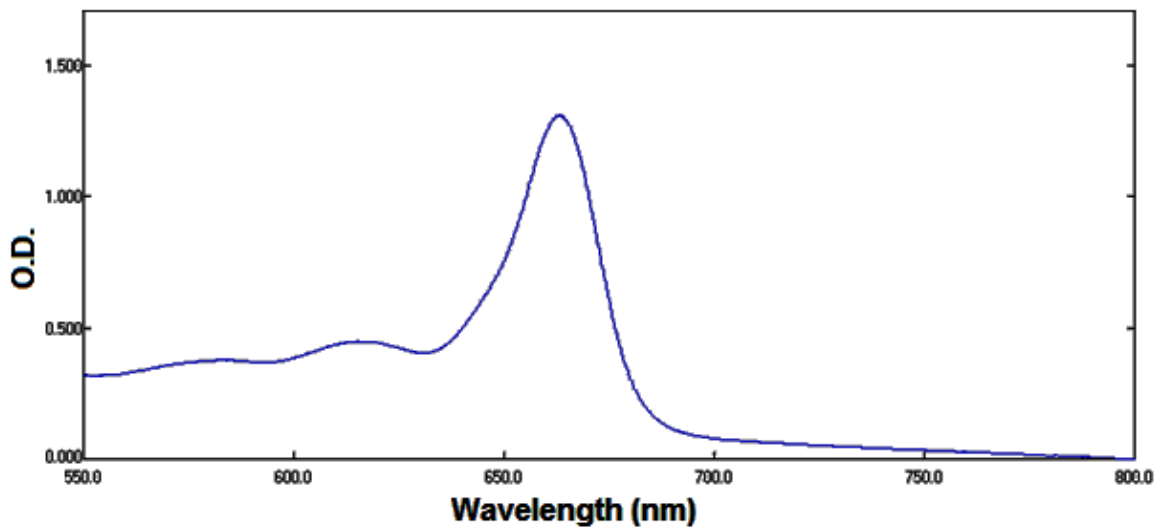
Figure 3.6 Spectrum of chlorophyll solution.

In Figure 3.7, the effect of atrazine, Cu^{2+}, phenol and sodium hypochlorite on chloroplasts and Hill reaction was evaluated. More specifically, the toxic effect of atrazine was monitored between the concentrations of 10^{-4}M and 10^{-7}M which correspond to 21.6mg/l and 2.2×10^{-2}mg/l. Atrazine inhibited the Hill reaction at the most concentrated samples and followed a typical sigmoid pattern. As the dilution rate increased, DPIP was reduced and its colour turned to colourless, meaning that lower concentrations of atrazine were not toxic to chloroplasts. Furthermore, the O.D. values were significantly deteriorated after both 5 and 10 minutes of illumination with P values $P < 0.05$ and $P < 0.001$ respectively.

The rest of the tested compounds (copper ions, phenol and sodium hypochlorite) were much less toxic to chloroplasts since they provoke same O.D. deterioration as the control. However, copper induced a moderate response at the highest concentration tested (24.2mg/l) which was more discrete after 10 minutes of illumination.
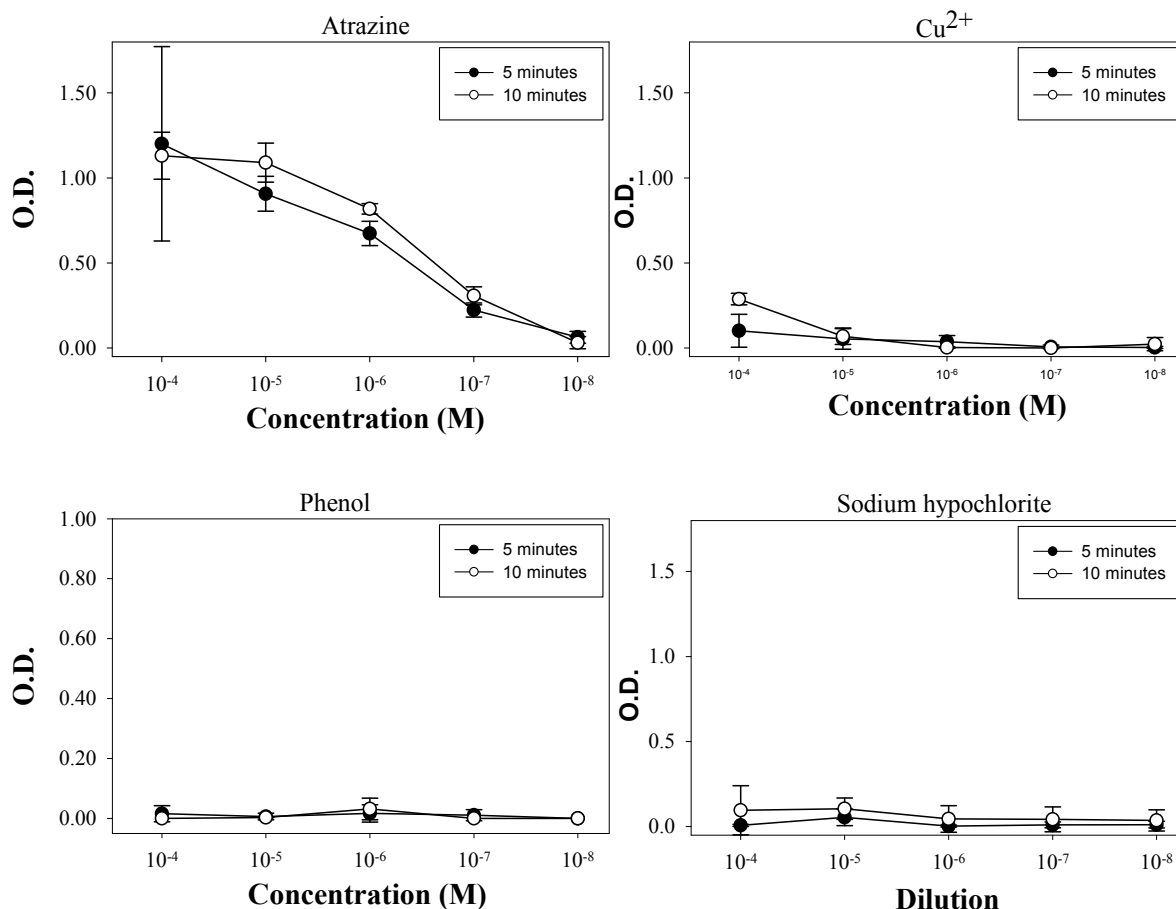
Figure 3.7 Absorbance at 550nm of the chloroplast suspension in the presence of atrazine, Cu^{2+}, phenol and sodium hypochlorite in relation to the concentration after 5 ($\bullet$) and 10 minutes ($\circ$) of illumination. The error bars represent the standard deviation of the mean of three replicates for each concentration.

3.4   Discussion

3.4.1 Discussion outline

The aim of this Chapter was to apply chloroplast assay to four contaminants frequently exist in wastewater. These compounds were also representatives of four main categories of chemical substances. The evaluation of their effect on the inhibition of the Hill reaction after two

specific illumination periods of 5 and 10 minutes was performed without any significant difference among the results. Furthermore, Lydon (2007) investigated the effect of illumination period on the absorbance of leachate samples and reported that after 15 minutes of illumination no further effect was recorded. Furthermore, there was no great difference between the absorbance values after 5 minutes of illumination and after 60 minutes of illumination period for undiluted leachate samples (Lydon, 2007).

Several problems faced during the performance of this assay including the stability of the chloroplasts which was poor. After several preliminary tests, the frozen chloroplast extract (-80°C), proved not as effective as the freshly extracted chloroplasts. Consequently, the chloroplast extract was prepared the day that it was going to be used. However, Piletskaya and co-workers (1999) reported that the thylakoid immobilisation in poly-(vinylalcohol) bearing styrylpyridinium groups (PVA-SbQ) improved the membrane stability and as a result the prepared test-system can be stored for one year at -20°C or for four months at +4°C.

3.4.2 Study of the effect of atrazine, copper ions, phenol and sodium hypochlorite on chloroplast assay

Based on the results of the application of chloroplast assay on atrazine, Cu^{2+}, phenol and sodium hypochlorite, it was testified that chloroplast assay constitutes a quite sensitive tool for detecting specific group of compounds. More specifically, the toxic effect of atrazine was monitored between the concentrations of 10^{-4} M and 10^{-7} M which correspond to 21.6 mg/l and 0.2×10^{-3} mg/l. However, according to Directive 2008/105/EC (2008) the maximum allowable concentration of atrazine in surface water is 2×10^{-3} mg/l. As a result, the chloroplast assay can successfully detect atrazine in low concentrations.

Regarding the toxic effect of copper ions, it was identified only at the highest tested concentration (24.2 mg/l) which is above the allowed limits of the UK government and the Water Quality Objectives (0.005-0.112 mg/l) (Dalley, 2001). Although, Ventrella (2007) indicated that copper ions can be successfully detected using chloroplast assay at the concentration range between 10^{-6} and 10^{-5} M, these results were not confirmed in this study. Unfortunately, chloroplast assay proved not suitable for the detection of phenol and sodium hypochlorite whatever the dilution.

3.5   Conclusions

The objective of this Chapter was to apply a plant cell toxicity assay, the chloroplast assay, in order to assess the toxicity of various compounds including atrazine, copper ions, phenol and sodium hypochlorite. These contaminants were selected since they commonly exist in water (groundwater, rivers, lakes, wastewater, etc.) and threaten the environment. The early-detection of potential contaminants would help the early-intervention and prevention of their spread.

The results of this chapter indicated that there is high potential of detection of herbicides and lower for heavy metals but there was not great sensitivity for the specific organic and inorganic compounds. Atrazine inhibited the Hill reaction, followed the expected sigmoid pattern and was successfully detected in very low concentrations. Furthermore, copper ions reduced chloroplast activity only at the highest tested concentration. But, the rest tested compounds, phenol and sodium hypochlorite, did not impair the Hill reaction.

Chapter 4 Microbial bioassays

4.1 Objectives

The objective of this part of the study was to assess the toxicity level of specific contaminants by applying two different endpoints of assays using bacteria. The first assay was based on the growth inhibition of *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* using the disc susceptibility assay to estimate the toxic effect of the contaminants. Atrazine, copper, phenol and sodium hypochlorite were analysed using this assay.

The second assay applied was the estimation of bioluminescence released by *Vibrio fischeri*. These bacteria emit light naturally, but in the presence of toxic agents, the level of luminescence decreases. The diminution of bioluminescence was determined using freshly prepared *V.fischeri* cells and ToxAlert® technology (Merck, Germany) for measuring the light output. However, only sodium hypochlorite and phenol were tested with this assay, due to problems encountered with the activity of the cells, whose luminescence activity became sporadic and unreliable. Finally, the growth curve of *V.fischeri* was characterized, in order to better understand the assay.

4.1.1 Disc Susceptibility assay

This assay is usually employed to estimate the susceptibility of various microorganisms, primarily bacteria, to antibiotics, disinfectants and antiseptics. Agar plates are utilised with the appropriate inoculum of bacteria. Then, filter paper discs are saturated with the chemical compounds in various concentrations and placed in the middle of the agar plates. If the organisms are susceptible to the compound on the disc paper, they will not grow around the disc. This area is called the "zone of inhibition" and is measured and compared to

standardised tables (Duncan, 2005). Several microorganisms were used during these experiments: *Escherichia coli*; *Pseudomonas aeruginosa*; and *Staphylococcus aureus*.

Escherichia coli is a Gram-negative, facultative anaerobic and rod-shaped bacterium. Some of the strains of *E. coli* are inhabitants of the human intestinal tract; prohibit the growth of harmful bacteria and help in the absorption of vitamins (Hu, 2002). On the other side, there are some strains of *E. coli* that are detrimental for human health and found in water and soil (Hu, 2002).

Pseudomonas aeruginosa is an aerobic, Gram-negative and rod-shaped bacterium. These bacteria commonly occur in soil, water, on the surfaces of the plants and animals and in the hospitals. Furthermore, they are better known as plant pathogens than animals and there are three strains that are pathogens of humans. In addition, *Pseudomonas aeruginosa* causes many infections such as gastrointestinal tract infections, dermatitis, respiratory infections, urinary tract infections and many more (Todar, 2008).

Additionally, *Staphylococcus aureus* is Gram-positive, facultative anaerobic coccus (Todar, 2008). These bacteria commonly exist on human skin and mucosa and can infect humans causing community-acquired and nosocomial infections (Lindsay and Holden, 2004). In particular, if they enter the body, they can cause urinary tract infections, skin and wound infections, bacteraemia and pneumonia. Finally, *Staphylococcus aureus* can cause food poisoning

The aforementioned organisms have been found in wastewater treatment plants. Subsequently, their application for estimating (a) their susceptibility to various toxicants and (b) their growth inhibition in non- and ECP-treated wastewater samples (Chapter 6) was examined.

4.1.2 Bioluminescence inhibition assay– *V.fischeri*

The key principal of the bioluminescence inhibition assay is the application of *Vibrio fischeri* or *Photobacterium phosphoreum* as test microorganism which naturally emits bioluminescence (Parvez, 2008). Advanced technologies have been developed for measuring the decrease of luminescence caused by toxic aqueous solutions.

In more detail, *V.fischeri* is a marine bacterium which preferentially exists in sub-tropical and temperate waters (Scheerer *et al.,* 2006). It is found either as free-living-planktonic organism or as symbiotic one on squid or certain fish. It naturally emits bioluminescence above a critical threshold which is a form of chemiluminescence catalysed by an enzyme (Scheerer *et al.,* 2006). Although initial absorption of photons is the main principal of light emission in fluorescence and phosphorescence, in bioluminescence and especially in *V.fischeri* another mechanism occurs.

More specifically, there are two substrates,

    a. luciferin, a reduced flavin mononucleotide (FMNH$_2$) and

    b. a fatty aldehyde (RCH, 7-16 carbons) (Scheerer et al., 2006).

The reduction of FMN (flavin mononucleotide) to FMNH$_2$ is catalysed by an external reductant which acts via flavin mono-oxygenase oxidoreductase (Equation 4.1).

$$NAD(P)H + H + FMN \xrightarrow{\text{Flavin reductase}} NAD(P) + FMNH_2 \qquad (4.1)$$
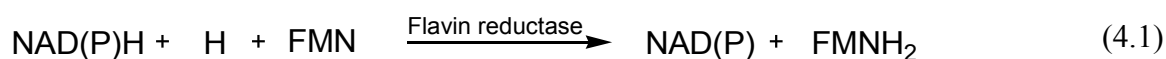
A 4a-peroxy-flavin intermediate is formed when the enzyme is bound to the reduced flavin (FMNH2) and reacts with oxygen. The acid (RCOOH) and an intermediate of luciferase-hydroxyflavin, in its excited state, are produced from the oxidisation of the aldehyde by the complex of 4a-peroxy-flavin intermediate. Subsequently, the excited state of luciferase-hydrohyflavin intermediate decays to the ground state and the emission of blue-green light follows at approximately 490nm (Equation 4.2) (Scheerer *et al.,* 2006).

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + H_2O + RCOOH + h\nu \ (490nm)$$

$$(4.2)$$
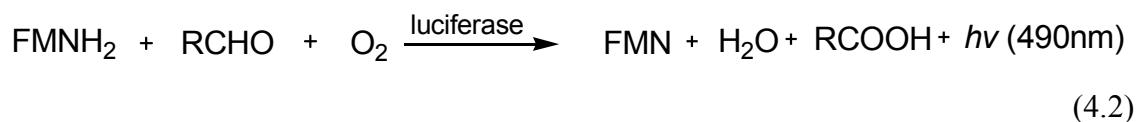
The decrease of the light output is evaluated by a luminometer. However, the light intensity output is dependent on several conditions such as salinity, temperature, pH and concentration of the toxicant (Broers and Lappalainen, 2004). The effective concentration of a toxic substance that causes 50% decrease of the emitted light during the nominated time intervals at 15°C is defined as EC_{50}. This assay is simple, rapid, reproducible and sensitive. Since it uses a marine bacterium for estimating the acute toxicity, it might not be representative for measuring the toxicity of fresh water ecosystems. However, many studies have been conducted using *V. fischeri* including the evaluation of toxicity of polycyclic aromatic hydrocarbons (El-Alawi *et al.,* 2002), organic pollutants present in wastewater treatment plants (Farré and Barceló, 2003) and the treated, using electrochemical oxidation, olive mill wastewater (Giannis *et al.,* 2007).

Many devices have been implemented in the last decades including LUMIStox[®] (Beckman Instruments), Microtox[®] (SDI), ToxAlert[®] (Merck, Germany) and AbraTox Kit[®] (Abraxis, LLC) that usually applied with freeze-dried *V.fischeri*. ToxAlert[®] assay employs free-dried bacteria which need reconstitution within 15 minutes and the light emitted is measured after 15 and 30 minutes of the addition of the toxicant. Then, the 50% of the light deterioration caused by the specific concentration of the toxicant was estimated (EC_{50}). The light output of the control (saline solution) is compared to the light output of the sample containing the toxicant and the inhibition percentage is determined (%I) using the following equation (4.3)

$$\% \text{ I} = [\text{ 1- (sample light/ control light)}] \text{ x } 100 \quad \textit{(Farré, 2003)} \quad (4.3)$$

However, the freeze-dried bacteria can be stored in a freezer for up to one year (Broers and Lappalainen, 2004). Although, they are ready-to-be-used, their shipment is rather expensive since they need to be kept frozen, usually using dry ice, during transportation and this is often problematic due to air transport restrictions (Broers and Lappalainen, 2004). Furthermore, their exposure to ambient temperatures could be detrimental for their quality and efficiency. Another option, especially when many samples would be analysed is the use of freshly prepared bacteria (ISO 11348-1:1999). A drawback of this approach, though, is the susceptibility of the bacteria to the cultivation method and the cell density of the batches.

Finally, *V.fischeri* bacteria require a thermostat during the conduction of the experiment since the appropriate exposure temperature is 15 °C (Broers and Lappalainen, 2004).

4.1.3 Microbial growth

Bacteria are prokaryotic organisms which mainly reproduce by binary fission (Bitton, 2005). During this process, when one cell grows up to its double size is dichotomised to two daughter cells. The same procedure is followed for all the cells and the microbial population exponentially increases. When an appropriate medium is inoculated with microorganisms, the growth of the population follows the pattern of the curve presented in Figure 4.1
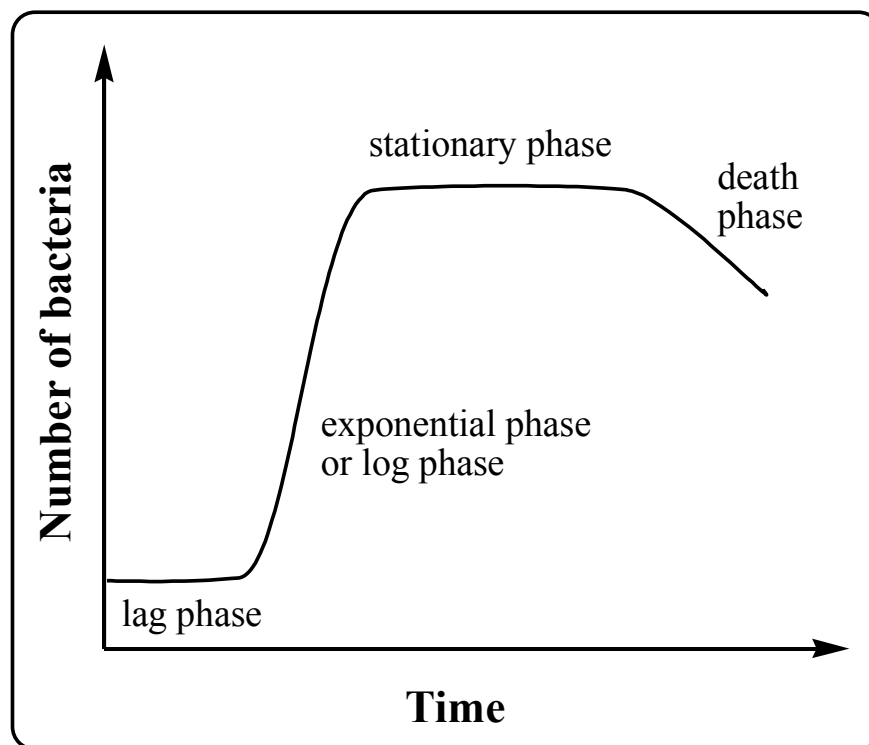
Figure 4.1 Microbial growth curve

During the *lag phase*, the cells are adapted to the new environment. The synthesis of biochemicals starts and the size of the cells increases. The length of this phase varies and depends on the cells prior history such as their age, the culture medium or their prior display

to damaging chemical and/or physical factors (Bitton, 2005). The exponential growth phase (or log phase) follows and the bacteria start reproducing exponentially. This stage is dependent on the growth conditions including temperature, pH and the composition of the medium. Then, the stationary phase occurs when the cells stop reproducing due to lack of nutrients and/or the accumulation of toxic metabolites that have been produced (Bitton, 2005). The last phase is the death phase where the death rate of the bacteria is greater than the growth rate (Bitton, 2005).

4.2   Materials and Methods

4.2.1 Disc Susceptibility assay

Reagents

The following reagents were used for the experiments and were of general laboratory grade unless stated otherwise:

Phenol was obtained from Sigma-Aldrich (Gillingham, UK). Hydrochloric acid, dimethylformamide (DMF) and copper nitrate [$Cu(NO_3)_2$ x $3H_2O$] were provided by Fisher Scientific (Loughborough, UK). Sodium hydroxide was obtained from Acros Organics (Loughborough, UK) and atrazine from Riedel-de Haen (Hanover, Germany). The sodium hypochlorite used was from the commercially available bleach (Domestos, Uniliever, UK). Finally, Milli Q water was obtained from Millipore (Watford, UK).

Culture of Microorganisms

Distinct colonies of *E.coli, P.aeruginosa* and *S.aureus* from the Cranfield Health laboratory culture collection were transferred to 50ml of liquid nutrient broth each (Oxoid, Basingstoke, UK) and incubated for 24 hours at 37 °C under stirring conditions. Then, ten-fold serial dilutions were performed for all the strains (10^{-1}-10^{-8}) under aseptic conditions. Finally, 0.1ml of each inoculum was spread in a labelled Petri dish with nutrient agar (Oxoid, Basingstoke, UK) for every species in duplicate and incubated at 37°C for 24 hours.

Disc susceptibility assay

Nutrient agar Petri dishes were inoculated with 0.1ml of *E. coli, P. aeruginosa* and *S. aureus* of 10^7 cells per ml, 10^8 cells per ml and 10^7 cells per ml respectively in order to obtain the ideal growth of the microorganisms for performing the assay (Colour plate 4.1). Sterilised paper discs (42.5mm, Whatman plc, Kent, UK) were saturated with the proper concentration of toxic compound and placed in the middle of the Petri dish. Then, the Petri dishes were incubated at 37°C for 24 hours. After 24 hours of incubation, the inhibition zones were measured.
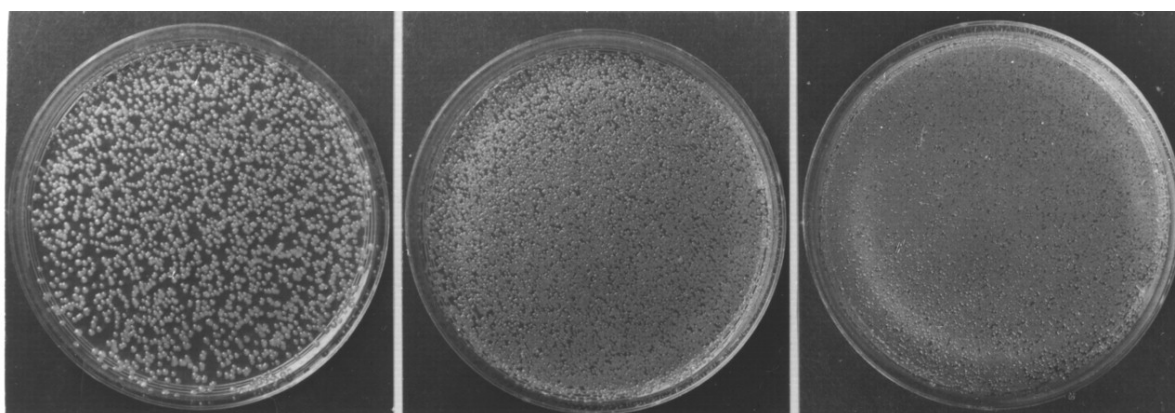
| Lightest acceptable | Ideal | Heaviest acceptable |

Colour plate 4.1 Bacterial growth (Adapted from Andrews, 2005)

4.2.2 Study of the effect of various toxic compounds on the growth of microorganisms

Atrazine (herbicide), copper (heavy metal), phenol (organic compound) and sodium hypochlorite (inorganic compound) were applied in various concentrations (Table 4.1) in order to estimate the inhibitory effect on the growth of specific microorganisms including *E. coli, P. aeruginosa* and *S. aureus.* 0.1ml of the appropriate inoculation (10^7 cells per ml) of each microorganism species were spread onto agar Petri dishes and then, the disc was dipped in the appropriate dilution of toxic material and placed on the agar plate. The disc should be placed a short period after the cultivation of the microorganism in order to verify that the bacterial growth has not started. The experiment was conducted in duplicate and all the Petri dishes were incubated at 37 ºC for 24 hours.

Table 4.1 Tested contaminants in the respective concentrations.

Contaminants	Concentrations (M or mg/l)
Atrazine	10^{-4}-10^{-8} or $21.6*10^0$ - $21.6*10^{-4}$
Copper ions	10^{-1} - 10^{-5} or $24.16*10^3$ - $24.16*10^{-1}$
Phenol	10^0-10^{-5} or $94.11*10^3$ - $94.11*10^{-2}$
Sodium hypochlorite*	10^0-10^{-5}

*measured as dilution, not absolute concentration

4.2.3 Bioluminescence inhibition assay– *V.fischeri*

The experimental procedure as described in BS EN ISO 11348-1:1999 'Water quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test)' was followed, with some modifications, which are included in my protocols.

Reagents

The same reagents were applied and were of general laboratory grade. The pH of the tested solutions was estimated and further adjusted with hydrochloric acid (1M HCl) or sodium hydroxide (1M NaOH) to pH 7.0 ± 0.2 using a pH-meter, model HI8014, Hanna instruments, (Leighton Buzzard, UK). 2% NaCl was used for the preparation of serial dilutions of the toxicants. Laboratory cultured *V.fischeri* strains (NRRL B-11177) were used for the experiments.

Agar medium

Agar medium was used for storage and preservation of *V.fischeri* cells. Agar medium is composed of the following constitutes: 30g sodium chloride (NaCl), 6.10g sodium dihydrogen phosphate monohydrated (NaH$_2$PO$_4$·H$_2$O) (BDH, Lutterworth, UK) , 2.75g dipotassium hydrogenphosphate trihydrate (K$_2$HPO$_4$·3H$_2$O) (BDH, Lutterworth, UK), 0.204g magnesium sulphate heptahydrate (MgSO$_4$·7H$_2$O) (BDH, Lutterworth, UK), 0.5g diammonium hydrogenphosphate [(NH$_4$)$_2$HPO$_4$] (Acros Organics, Loughborough, UK), 3ml glycerol, 5g bacteriological peptone (LAB M, Lancashire, UK) and 0.5g yeast extract (Sigma Aldrich, Gillingham, UK), dissolved in distilled water to total volume of 1l. The pH was adjusted to 7.0 ± 0.2 with 0.1M NaOH or 0.1M HCl. 12g agar (OXOID, Hampshire, UK) were added and the medium was sterilised in an autoclave (121ºC, 20min) and transferred to Petri dishes. All the reagents were of general laboratory grade. The agar plates were stored at 5°C.

Liquid broth

Liquid broth for *V.fischeri* was prepared with the same reagents as agar medium but without agar. The pH was adjusted to 7.0 ± 0.2 with 0.1M NaOH or 0.1M HCl and made up to 1l

litre with distilled water. Then, 50ml of the mixture were transferred to 250ml Erlenmeyer flasks and sterilised in the autoclave (121ºC, 20min).

Solution for freshly prepared *V.fischeri*

This solution was prepared using 7.273g/l D(+)-Glucose ($C_6H_{12}O_6$), 20g/l NaCl, 2.035g/l magnesium chloride hexahydrate $MgCl_2 \cdot 6H_2O$, 0.30g/l potassium chloride (KCl) and 11.9g/l N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic) acid (HEPES). All the reagents were of general laboratory grade. The mixture was dissolved in distilled water, stirred for 30min and the pH was adjusted to 7.0 ± 0.2 with 0.1M NaOH or 0.1M HCl. Finally, it was made up to one litre with distilled water. This solution was stored in 50ml tubes at -20 ºC.

Cultivation of *V.fischeri*

Frozen cultures of *V.fischeri* were transferred to Petri dishes with agar medium under sterile conditions. They were incubated for 2 days at 20 ºC and then were stored in the fridge (4 ºC) for a maximum period of two weeks. After this period, the luminescent colonies were transferred to fresh Petri dishes with agar medium in order to preserve a continuous supply of *V.fischeri* (Colour plate 4.2).
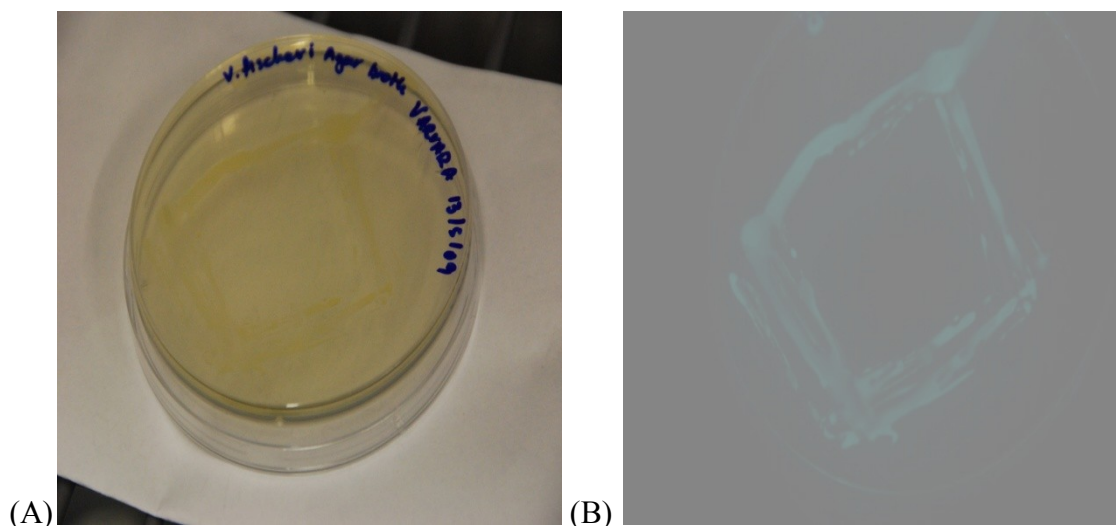
Colour plate 4.2 *V.fischeri* cultivation in the light (A) and in the dark (B).

Protective medium

This medium was prepared in order to protect the bacterial cells during storage in the freezer by dissolving 66g D(+)-Glucose, 4g NaCl, 2g L-Histidine and 0.5g Bovine Serum Albumin (BSA) at 37 ℃ in 1l of solution. The pH was adjusted with 1M HCl or 1M NaOH to pH to 7.0 ± 0.2.

Pre-cultures preparation

After two days of incubation, luminescent colonies of *V.fischeri* were inoculated in 50ml of liquid broth for 21 ± 1 hour at 20 ℃ under shaking conditions (180 r/min). Turbidity levels were determined of 1:10 dilution in NaCl solution using a spectrophotometer (CamScan350) at 578nm.

Main culture preparation

The main culture was prepared by inoculating 1ml of the pre-culture to 50ml of the liquid broth in a 250 ml Erlenmeyer flask (Colour plate 4.3). The mixture was incubated for 20 ± 1 hour at 20 ℃ under shaking conditions (180 r/min). Turbidity levels were determined of 1:10 dilution in NaCl solution using a spectrophotometer (CamScan350) at 578nm.
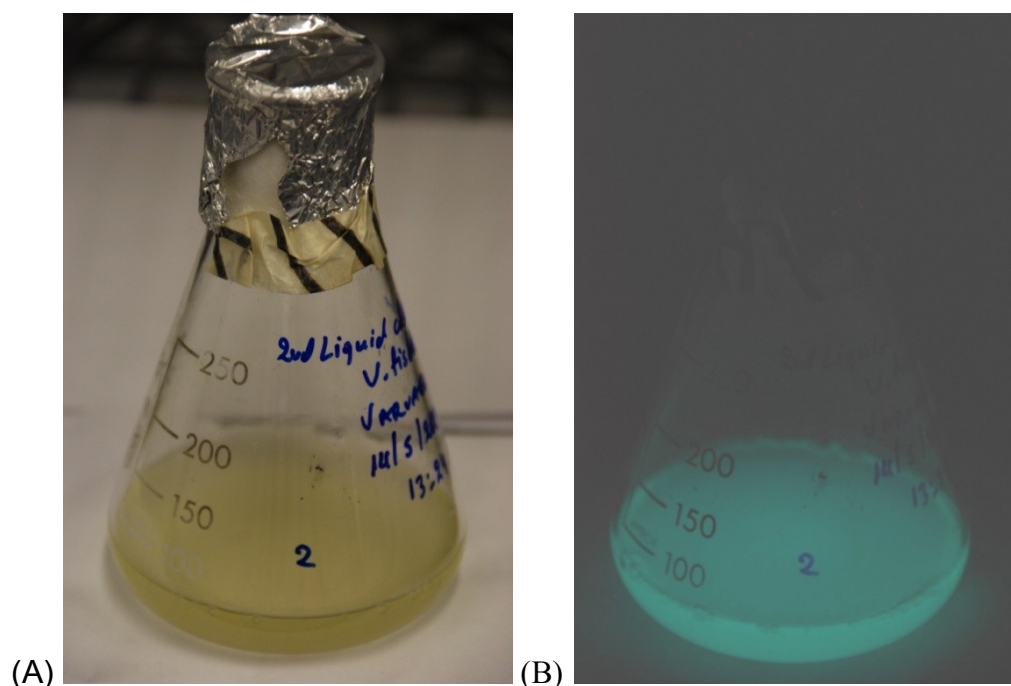
Colour plate 4.3 Liquid culture of *V.fischeri* in the light (A) and the same culture in the dark (B) showing the emission of bioluminescence.

Stock suspension preparation

Centrifugation of the bacterial suspension from the main cultures in a pre-cooled centrifugation Sorvall RC5C (DuPont Company, Delaware, USA) at 4 ℃ $\pm$ 2 ℃ was performed for 20 min at 6000 g. The supernatant was decanted; the sediments were re-suspended with ice-cold 2% NaCl solution (5-10 ml/ 50 ml of main culture) and centrifuged again. The same process repeated with decantation of the supernatant and re-suspension of

the pellets with ice-cold 2% NaCl solution (0.5-10 ml/ 50 ml of main culture). Then, the bacterial suspension was transferred to a pre-cooled beaker and placed on ice. 4ml protective medium per 50 ml of main culture was added slowly and under constant stirring. Determination of turbidity was performed of a 1:100 dilution with NaCl at 578 nm. 10 ml per 1ml protective medium was added and stirred for 15 minutes in order to obtain a homogenous mixture.

Procedure for analysing the samples

Preparation of serial dilutions of the toxic substances in NaCl and adjustment of the pH between 6 and 8.5 were initially performed. Sodium chloride solution was used for control samples and preserved at 15 ºC. 0.5 ml of the solution for freshly prepared *V.fischeri* cells per 100μl stock suspension were added to the *V.fischeri* stock suspension. This mixture was homogenised by agitation and maintained at 15°C for 15 minutes.

The followed procedure was according to ToxAlert[®] MULT assay (Merck KgaA, Germany) (Appendix B). The instrument was adjusted according to the instructions. Sterilised tubes were placed in two rows, one row for controls (A) of the same number of samples and one row with the samples (B). After a period of 15 minutes that *V.fischeri* suspension was maintained at 15 ºC, all the tested tubes were filled with 0.5 ml of this mixture. Then, 0.5 ml NaCl was added to the first tube of row A and 15 minutes started counting. In the meantime and after 30 seconds from the addition of NaCl to the first tube, 0.5ml of the toxic sample was inserted into the first tube of the row B. After 1 minute in total, 0.5 ml NaCl was added to the second tube of the row A and after 1.5 minute 0.5ml of the toxic sample was inserted into the second test tube of the row B. The same procedure and in the same order was repeated for all the tested samples and their controls.

When the 15 minutes elapsed, the first tube from row A was loaded in the ToxAlert[®] measured and removed. After 15 minutes and 30 seconds, the first sample from row B was loaded, measured and removed. The same methodology was performed every 30 seconds for all the controls and the respective samples until all of them to be measured. Finally, after 30 minutes from the beginning of the process the samples were measured again with the same order every 30 seconds.

Growth curve of *V.fischeri*

Three main cultures were prepared and their optical density (O.D.) was measured after frequent time intervals. The aim was to determine the growth curve of *V.fischeri*. Subsequently, the O.D. was estimated using a spectrophotometer at 578 nm and also 100 μl of the inoculations at the specific time intervals were plated onto agar Petri dishes.

4.2.4 Study of the effect of various toxic compounds on *V.fischeri*

Sodium hypochlorite and phenol were applied in various concentrations (Table 4.2) in order to evaluate the bioluminescence inhibitory effect. Stock solutions of sodium hypochlorite (10^{-2} M) and phenol (10^{-2} M) with 2% NaCl were prepared and their pH was adjusted to 7.0 ± 1.0 with 1 M HCl or 1 M NaOH. Freshly prepared *V.fischeri* cells were produced for every toxic compound tested and the aforementioned procedure (section 4.2.3) was followed.

Table 4.2 Tested contaminants in the respective concentrations.

Contaminants	Concentration (M) and Dilution
Phenol	10^{-2}, 5×10^{-3}, 10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5}, 10^{-5}, 10^{-6}, 10^{-7}
Sodium hypochlorite*	10^{-2}, 5×10^{-3}, 10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5}, 10^{-5}, 10^{-6}, 10^{-7}

*measured as dilution, not absolute concentration

4.3   Results

4.3.1 Study of the effect of various toxic compounds on the growth of microorganisms

The results revealed that atrazine and copper salt dilutions did not inhibit the growth of the applied bacteria. The strains of *E. coli, P. aeruginosa* and *S. aureus* were relatively resistant to those chemicals even to the most concentrated samples. However, phenol inhibited the growth of all the three bacteria at 1 M but the other concentrations did not affect the bacteria. Finally, sodium hypochlorite killed all the bacteria at the most concentrated dilutions of 1 M and inhibited the growth at 0.1 M for all the tested strains (Table 4.3). The remaining dilutions did not inhibit the growth of the bacteria.

Table 4.3 Inhibition of growth of the tested toxicants to the applied microorganisms

Contaminants	Atrazine	Cu^{2+}	P h e n o l		Sodium hypochlorite*		
Concentration/ Dilution			1M	$\leq 10^{-1}$M	1	10^{-1}	$\leq 10^{-2}$
E.coli	NI**	NI	44.5-52.5mm	NI	NG***	49.5-57.5mm	NI
P.aeruginosa	NI	NI	52.5-56.5mm	NI	NG	61.5-83.5mm	NI
S.aureus	NI	NI	52.5-72.5mm	NI	NG	56.5-86.5mm	NI

*measured as dilution, not absolute concentration
**NI: No Inhibition
***NG: No Growth

4.3.2 Bioluminescence inhibition assay– *V.fischeri*

Although four contaminants were usually applied for the rest of the toxicity assays of this study, only two (sodium hypochlorite and phenol) were examined using *Vibrio fischeri* cells. The *V.fischeri* assay proved difficult to calibrate reliably with freshly prepared cells. The obtained results for both sodium hypochlorite and phenol are further described in the following section (4.3.3). Furthermore, the characterisation of the growth curve of *V.fischeri* was also performed. However, *V.fischeri* cells were unexpectedly interrupted emitting bioluminescence and none of the attempts to culture them again from stock cultures was successful. Consequently, the remaining toxic compounds, atrazine and copper salts, were not analysed.

4.3.3 Study of the effect of various toxic compounds on *V.fischeri*

Stock solutions of sodium hypochlorite (10^{-2} dilution) and phenol (10^{-2} M) with 2% NaCl were prepared and their pH was adjusted to 7.0 ± 1.0. Various dilutions were performed for both contaminants and the results are displayed in the following tables (Table 4.4 and 4.5).

Table 4.4 Percentage inhibition after 15 and 30 minutes of exposure of *V.fischeri* cells to sodium hypochlorite solutions.

Dilution	10^{-2}	5×10^{-3}	10^{-3}	5×10^{-4}	10^{-4}	5×10^{-5}	10^{-5}	10^{-6}	10^{-7}
% Inhibition									
$\%I_{15}$	←				Light too high[*]				→
$\%I_{30}$	←				Light too high				→

[*] Above the machine's threshold

Table 4.5 Percentage inhibition after 15 and 30 minutes of exposure of *V.fischeri* cells to phenol solutions.

Concentration (M)	10^{-2}	5×10^{-3}	10^{-3}	5×10^{-4}	10^{-4}	5×10^{-5}	10^{-5}	10^{-6}	10^{-7}
% Inhibition									
$\%I_{15}$	0	16	0	25	0	0	0	0	3
$\%I_{30}$	0	13	0	24	0	0	0	0	5

As presented in the Table 4.4, the light emission after 15 and 30 minutes was not inhibited by sodium hypochlorite. Subsequently, the applied samples were not toxic enough to decrease the luminescence of *V.fischeri* cells. Regarding phenol solutions, inconsistent results were observed (Table 4.5). Bioluminescence inhibition observed in low percentages at the samples of 5×10^{-3}, 5×10^{-4} and 10^{-7} M.

Since phenol results were quite inconsistent, the characterisation of the *V.fischeri* growth curve was performed. According to the experimental procedure, the growth of *V.fischeri* main culture was interrupted after 20 hours of constant stirring at 20 °C. Subsequently, an inoculation was prepared in the same way as described for the main culture (section 4.2.3) and the measurements of O.D. using a spectrophotometer at 578nm started after 3 hours (1st sample) and then for frequent time intervals. Two more inoculations were also performed and the measurements began after 11 and 16 hours (2nd and 3rd sample respectively) for defining the transition from the lag to the exponential phase and also to testify if the results are reproducible. The results are illustrated in the following graph (Figure 4.2).
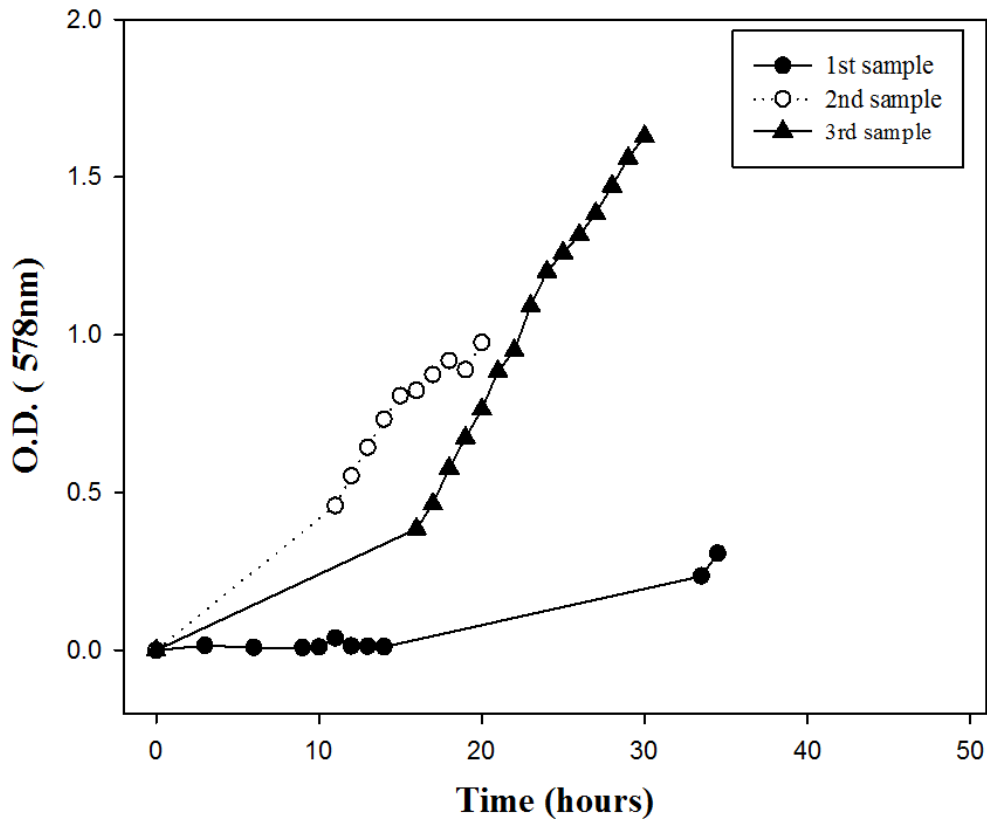
Figure 4.2 Growth curves of *V.fischeri*,three replicates.

The curve from the first sample showed that the exponential stage was quite delayed and started after 33 hours (Figure 4.2). However, the exponential phase according to the curves of the 2nd and 3rd samples had already started before the first measurements of O.D. were performed. Consequently, the bacteria had started to reproduce before the 20th hour and continued for several hours according to the second and third inoculation. Finally, the plate counts did not result in distinct colonies but swarming for all the applied dilutions.

Unfortunately, at this stage *V.fischeri* cells stopped emitting luminescence and further attempts with stock cultures were not successful.

4.4   Discussion

4.4.1   Disc Susceptibility assay

The disc susceptibility assay is based on the diffusion of the toxicant/drug to the agar medium. A successful performance of this assay is leaned upon the simultaneous inoculation of the microorganism and the placement of the disc. This assay also constitutes a qualitative method to identify potential toxic solutions. However, many factors can affect the produced results such as the type of the agar and its thickness in the plates, the bacteria growth rate, the amount of the toxicant on the disc, the size of the disc and the diffusion rate of the contaminant (Lorian, 2005).

4.4.2   Study of the effect of various toxic compounds on the growth of microorganisms

The bacteria applied, *E. coli, P. aeruginosa* and *S. aureus,* for estimating their growth inhibition using the disc susceptibility assay proved relatively resistant to the toxic compounds of atrazine, Cu^{2+}, phenol and sodium hypochlorite. The most concentrated samples of phenol and sodium hypochlorite impaired the microorganisms and the zones of inhibition were obvious. On the other side, atrazine and Cu^{2+} did not affect the growth of the bacteria at any of the concentrations used. However, the potential occurrence of the specific strains in activated sludge justifies their resistance.

4.4.3   Bioluminescence inhibition assay– *V.fischeri*

The performance of the bioluminescent assay using *V.fischeri* and ToxAlert® proved difficult for several reasons. Firstly, the growth of *V.fischeri* was not consistent and/or reproducible. Furthermore, ToxAlert® is normally applied with freeze-dried bacteria which

only need reconstitution. During these experiments, freshly prepared cells were utilised since stock cultures existed in Cranfield Health laboratory. However, a sudden termination of bioluminescence emission from *V.fischeri* cultures led to interruption of the experiments. This incident occurred due to the growth of the bacterial cells in a very diluted or unconfined environment (Nealson, 1977). Subsequently, the inducer was probably not accumulated to the appropriate concentration of induction and luminous system was depressed and became dark (Nealson, 1977). Further attempts to culture the bacteria from stock cultures were performed but with no success.

4.4.4 Study of the effect of various toxic compounds on *V.fischeri*

Many preliminary assays were performed in order to achieve the optimisation of the procedure and start obtaining results. The usual contaminants that were applied to all the toxicity assays of this study were not all tested. Sodium hypochlorite and phenol were the only toxic substances that were applied with *V.fischeri*.

The initial step performed for the tested compounds was the pH adjustment. According to Isidori and colleagues (2003), the toxicity is associated with pH and more specifically the toxicity increases when the pH values are higher than the appropriate levels and declines in opposite conditions. So, the pH of the samples should range between 6 and 8.5

Furthermore, in accordance to the obtained results, sodium hypochlorite did not significantly impair the bioluminescent emission. The light levels were too high for ToxAlert® to estimate, meaning that the samples were not toxic enough to affect the light output. Furthermore, phenol solutions provoked a measuring output for ToxAlert® but it was very low, meaning again that the tested phenol samples were not very toxic to *V.fischeri*. As a result, the tested samples were either not toxic or much diluted to influence the emitted bioluminescence.

In order to justify the results, plate counts were conducted and the growth curve of *V.fischeri* was characterised. Although, the experiment was performed three times, the results showed that the exponential phase is not standardised. The BS EN ISO (11348-1:1999) recommends the interruption of the growth of *V.fischeri* cells after 20 hours of

incubation. Regarding the achieved results from the growth curve, the bacteria lied upon the exponential phase after 20 hours of incubation. Consequently, the phase of their evolution that they were applied was the proper one for achieving high bioluminescence levels.

4.5   Conclusions

In conclusion, this chapter aimed to assess the toxic effect of several toxic compounds on growth inhibition (*E. coli, P. aeruginosa* and *S. aureus*) and bioluminescence deterioration (*V.fischeri*).

Regarding the first assay, the tested microorganisms were not susceptible to atrazine and copper salts but were sensitive to the most concentrated samples of phenol (1 M) and sodium hypochlorite (1 and 0.1 dilution). Since these microorganisms are found to wastewater, were further applied in Chapter 6 as a process indicator of the toxicity of wastewater before entering the wastewater treatment plant.

On the other side, the toxic substances applied to *V.fischeri* cells were sodium hypochlorite and phenol due to unexpected interruption of bioluminescence emission. The examined samples had no significant deterioration on the emission of bioluminescence. The light levels were above the machine's threshold for sodium hypochlorite samples which were in accordance to the results obtained by the disc susceptibility assay for the same concentrations since the bacterial growth was not inhibited at the specific concentrations. Additionally, the same concentrations of phenol samples were applied for estimating their toxicity to the growth inhibition assay and to the bioluminescent inhibition assay. All of the samples caused the same effect and there was no inhibition for both assays. However, the growth curve of *V.fischeri* did not show any particular consistency which explains the unstable results for phenol samples.

Chapter 5 Invertebrate toxicity assays

5.1   Introduction to aquatic toxicology and invertebrate toxicity assays

According to the journal of the same name, Aquatic Toxicology refers to: "the mechanisms of toxicity in aquatic environments and the understanding of responses to toxic agents at community, species, tissue, cellular and subcellular level, including aspects of uptake, metabolism and excretion of toxicants; understanding effects of toxic substances on aquatic ecosystems; toxicant-induced alterations in organisms as evidenced, for example, through biochemical and physiological reactions, including adaptive responses; the development of procedures and techniques that significantly advance the understanding of processes and events that produce toxic effects; in-depth studies of human health aspects of aquatic toxicology. Chemical and other identification of toxicants will be considered when related to the understanding of perturbations in life processes" (Nikinmaa and Schlenk, 2011).

From the perspective of this project, the scope is particularly focused on the often disastrous consequences on living organisms in the aquatic environment from the use of chemicals by humans (Kendall *et al.,* 2001). In addition to the primary effects, the water contamination travels through the food chain and reaches higher species of life with undesirable effects, often exacerbated by bioaccumulation. Therefore, the early detection of contamination is imperative for protecting life.

As a result, many assays for assessing toxicity, using various organisms, have been developed. These are based on a variety of species, including microorganisms, invertebrates, algae and fish. However, in this study, an assay using each category has been applied, apart from fish. The latter are more problematic for this purpose for several reasons, including ethical issues and the need for a licence to work with vertebrates. A suitable intermediate between chloroplasts and bacteria, and fish, is the use of an invertebrate species. One of the most common organisms applied to such assays is *Artemia* species. *Artemia*, as a testing organism for assessing toxicity, has several advantages that will be further discussed in the following section.

However, the objectives of the experiments described in this chapter were:

- The application of an existing assay using *Artemia salina* to assess the toxic effect of various compounds in aqueous solutions.
- The development of a novel device for early detection and warning of potential toxic compounds in aqueous solutions.
- The optimisation of the performance of this instrument.
- The comparison of the results to the existing ARC-test (Artemia Reference Centre).
- The application of the device to a wide range of toxicants and mixtures of them.
- The development of a protocol that could be applied with the newly developed device and that can perform as a screening tool for identifying potentially toxic solutions.

5.1.1 General characteristics of *Artemia* species

Artemia species or brine shrimps (i.e. *A. salina*, *A. franciscana, A. parthenogenetica*) belong to the phylum Arthropoda and to class Crustacea and are broadly used for toxicity studies (Sarabia *et al.,* 2006). They can survive in a wide range of salinity levels from fresh water to 300 g/l of salinity. However, in fresh water they are not reproductive (Treece, 2000). Artemia species can be found in lakes with high salinity levels such as in Great Salt Lake in the northern part of Utah and in Mono Lake, California or in man-made salt pans or coastal lagoons (Nunes *et al.,* 2006). Furthermore, they withstand temperatures from 15 to 55°C (Treece, 2000) and pH levels higher than 7.5 (Vanhaecke and Persoone, 1984). *Artemia* cysts have a diameter of 200 to 300 μm dependent on the species. The dried cysts can be stored in containers for years without losing their ability to hatch (Treece, 2000). The nauplii are about 0.5mm in length and they can reach 15mm during their life cycle.

When the optimum conditions of oxygen, salinity and temperature exist, the hatching process begins. After a period of 15 to 20 hours maintenance of the *Artemia* cysts (Colour plate 5.1A) in artificial seawater and under a constant temperature above 25°C, the pre-nauplius in E-1 phase develops by breaking the cyst shell. This is the first phase or *umbrella* phase of the embryos. During this phase, the pre-nauplii have the cyst shell attached beneath them (Colour plate 5.1B). This stage is important because the pre-nauplii are fed from the shell yolk until their mouth will be well-developed. If the conditions

become unfavourable (i.e. rise of salinity), then the nauplii will be encased in a cyst and they will hatch again when the conditions improve. The next phase, Instar I nauplius, is when the pre-nauplius E-2 is detached from the shell. After around 12 hours, the nauplii turn into Instar II and they begin filter feeding on bacteria, detritus and microalgae (Treece, 2000). *Artemia* undergo fifteen instars in a period of eight days until entering adulthood. Barahona and Sánchez-Fortún (1996) have evaluated the sensitivity of different age specimens and they have reached the conclusion that the 48 hour old *Artemia* nauplii (stage instar II-III) showed higher relative sensitivity for the most of the tested substances (Colour plate 5.1C). Other end-points such as their behaviour or hatchability success or different age specimens or photatactic response have been also used with satisfactory results.
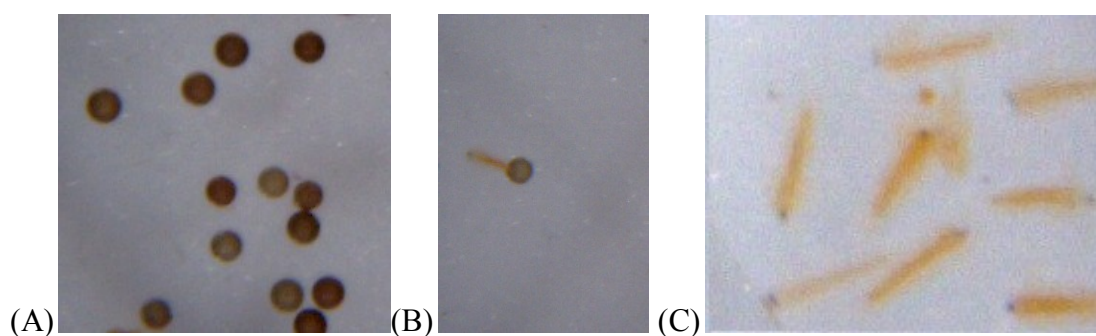
Colour plate 5.1 Artemia salina hatching stages from cysts (A), after 24 hours in stage instar I (B) and after 48-hours in stage Instar II-III (C).

5.1.2 Advantages and disadvantages of Artemia species

Artemia species offer several advantages over other test organisms used for toxicity assessment, including the following:

- ✓ Constant commercial availability all year round,
- ✓ Ease of culture,
- ✓ Short life-cycle,
- ✓ No need to feed them during the assay,
- ✓ Great offspring production
- ✓ No need of preserving live stock (Nunes *et al.,* 2006),

- ✓ Simple and quick bioassays regardless of the end-point measured,
- ✓ Cost efficient and
- ✓ Reproducible and accurate results

All the aforementioned advantages lead to a reliable bioassay (Vanhaecke and Persoone, 1984).

On the other side, Nunes and co-researchers (2006) have reported low sensitivity of Artemia species comparing to other crustacea such as *Daphnia magna*, *Daphnia pulex* and *Thamnocephalus platyurus*. Moreover, Nałęcz-Jawecki *et al.* (2003) have performed a comparative study of susceptibility to fifteen quaternary ammonium compounds among *Vibrio fischeri* (bacterium), Spirostomum *ambiguum* and *Tetrahymena thermophia* (protozoa), and *Artemia franciscana* (invertebrate). This study concluded that *Artemia franciscana* has the lowest sensitivity, compared to the other tested organisms. However, the endpoint usually estimated in these studies is the mortality of the nauplii. Consequently, the end-point applied for estimating the toxicity using *Artemia* species may be improved by using more sensitive end-points, which exist, such as the level of mobility of the nauplii.

5.1.3 Applications

The first attempt at analysing the *Artemia* cyst characteristics was performed by Sorgeloos (1980). A broad investigation across 80 American and European laboratories proved that an accurate and reliable assay for measuring the toxicity could be implemented (Persoone and Vanhaecke, 1981). Afterwards, Vanhaecke and Persoone (1984) standardised a toxicity assay based on *Artemia* cysts and developed the "Artemia Reference Centre"- test, the so called ARC-test. This is an acute toxicity bioassay based on the lethal effects of toxicants to 50% of the nauplii at the stage of Instar II-III after 24 hours of exposure to toxic substances and under specific conditions (24h-LC_{50}) (Vanhaecke and Persoone, 1984).

The ARC-test still constitutes the basic assay that has been used for many years in order to assess toxicity. Many contaminants have been tested using this bioassay, including pesticides (Baharona and Sanchez-Fortun, 1999), heavy metals (MacRae and Pandey, 1991), leachates (Svensson *et al.,* 2005), cytotoxicity of dental materials (Pelka *et al.,* 2000), fungal

toxins (Harwig and Scott, 1971) and antifouling biocides (Panagoula *et al.,* 2002; Koutsaftis and Aoyama, 2007).

Apart from using this assay, many researchers used other end-points of Artemia species in order to assess the toxicity. Firstly, the different age of the shrimps has been investigated by Barahona and Sánchez-Fortún (1996). These authors reached the conclusion that the most sensitive stage is the Instar II-III (48 hours old). The hatchability success has also been evaluated in comparison to the mortality of the nauplii. The results indicated high accordance, which translated to the need of further development of the two bioassays (Carballo *et al.,* 2002). Furthermore, the hatching success has also been investigated by exposing the Artemia cysts to heavy metals (MacRae and Pandey, 1991; Bagshaw *et al.,* 1986). These studies indicated that hatching success is a more sensitive end-point for measuring the metal toxicity since the EC$_{50}$ of Cd, Cu and Zn were comparable and sometimes lower than the values measured by United States Environmental Protection Agency (USEPA, 1987, 2001, 2003) (Brix *et al.,* 2006). Additionally, behavioural parameters such as the photo tactic response of the nauplii to environmental stressing substances were studied by Di Delupis and Rotondo (1988). Venkateswara and co-researchers (2007) studied the effect of LD$_{50}$ values of organophosphates on the morphology and locomotor behaviour of *Artemia* nauplii (48 hours old). Enzymatic disruptions such as cholinesterase inhibition of *A. salina* and *A. parthenogenetica* have also been researched to assess the toxicity of organophosphorous pesticides (Varó *et al.,* 2002 a and b; Nunes *et al.,* 2006). Finally, Portmann and co-workers (1996) have patented a device which can assess the toxicity of aqueous solutions by determining the change in mobility and/or size of the used organisms. Furthermore, Ethovision is a video tracking behavioural system for organisms moving in a specific arena and is commercially available. This software identifies the centre of gravity of each organism exist in the arena and evaluates its movement. It can estimate various parameters such as mean velocity for all animals undertaken a specific treatment, path shape and minimal distance covered. Ethovision has been applied to various animals for tracking their behaviour under the effect of a specific medicine. Most commonly, rats and mice have been tested while their brains have been altered by receiving neurochemicals or drugs (Noldus *et al.,* 2001). It has also been applied for estimating the locomotor behaviour (swimming velocity and distance covered per unit time) of *Artemia salina* nauplii in organophosphate solutions at the LC$_{50}$ concentrations after 24 hours of incubation (Venkateswara *et al.,* 2007).

However, another approach of the device patented by Portmann et al (1998) in conjunction with more specific software than the one of Ethovision has been implemented. This new developed device combines the advantages of the aforementioned technologies and also determines the 50% inhibitory concentration that provokes the deterioration of 50% of the movement ability of the nauplii comparing to controls. Additionally, this device can be used as screening tool for evaluating if a water sample is potentially toxic.

5.1.4 Development of a new device

The evolution of a new device started while observing the deterioration of the movement of the nauplii in a toxic environment. As a result, the main hypothesis was that the accumulation of toxicants to *Artemia salina* nauplii has the effect of reducing their mobility and this deterioration can be measured. The term "mobility" has been adopted from solid-state physics in order to describe the "movement activity" (Portmann *et al*., 1998). So, the main aim was to detect subtle changes in the mobility of the nauplii caused by contaminants in aqueous solutions and determine their toxicity by processing an image sequence. This image sequence would be recorded for each sample and processed using a newly-developed computer vision algorithm. This algorithm tracks the movement of the nauplii and estimates the distance covered in a specific period of time (Colour plate 5.2). The left image shows a representative video frame as captured by the camera. A sequence of such frames forms the input to the digital image processing algorithm. Although the shrimps are not clearly visible in the cuvette, they can be tracked with good accuracy in order to measure their mobility. Lighter gray shades denote areas of higher mobility including overlapping tracking paths (Kokkali *et al.,* 2010).
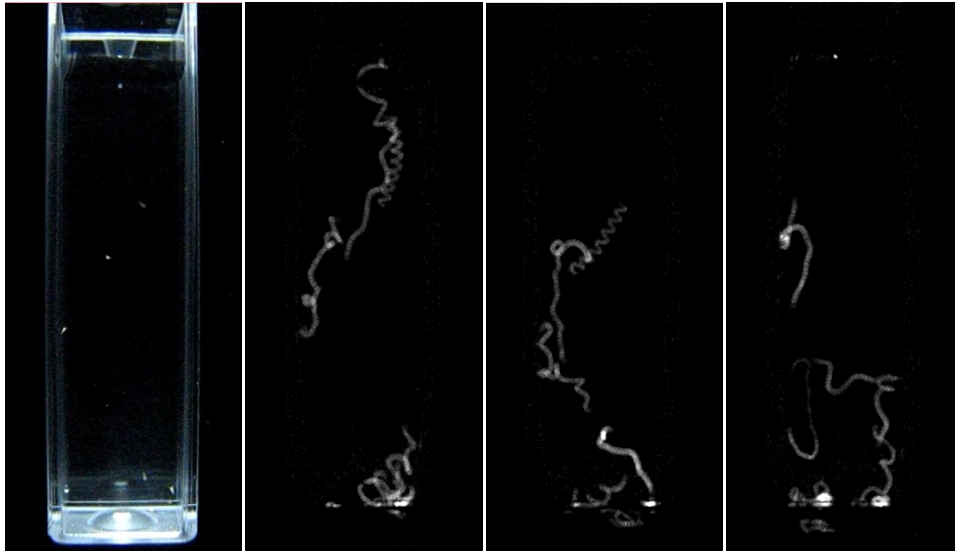
Colour plate 5.2 The left image shows a representative video frame as captured by the camera. The remaining images show some characteristic examples of tracking paths which were derived over 6.6 sec intervals.

The choice of *Artemia salina* as the tested organism was based on the fact that they live in water and their mobility can easily be monitored as described in the earlier section 5.1.2. Additionally, this species does not need to be fed during the assay.

A step-by-step iterative approach had to be adopted in order to derive the optimum configuration and protocols for operating this device. Firstly, the containers used were 10ml round vials (Colour plate 5.3). Videos were recorded of those with the 5ml toxic solution and 10 nauplii for 20 seconds in an open air environment. Atrazine, copper ions, phenol and sodium hypochlorite were initially tested. However, several weaknesses were identified while processing the videos via the algorithm. Primarily, the light reflections from the open air recordings increased the noise of the final measurements. Also, round vials proved suboptimal due to their high curvature which meant that certain parts of the vial were hidden from the camera view. Moreover, the recorded video length for this type of vials was very short to estimate the mobility of the nauplii. Nevertheless, the results from all the tested compounds verified the expected trend line and the recorded mobility was seen to be significantly reduced as the concentration of contaminants increased.

Colour plate 5.3 10 ml round vials.

After this encouraging step, the focus shifted on improving the performance of the system by:

a) reducing the amount of image noise,

b) replacing the round vials with a more suitable type and

c) increasing the recording time.

The first problem was solved by introducing a dark chamber attached to the camera lens with directional LED illumination. This configuration meant that all experiments could be performed under identical illumination conditions, thus reducing the amount of error caused by changes in the ambient light intensity and direction. Furthermore, the round vials were replaced by the 4.5 ml polystyrene disposable cuvettes. The rectangular shape of these cuvettes meant that its entire volume could be recorded with the camera which was not the case with the round vials. As a result, the movement of the nauplii could be monitored with minimal error using only one camera.

Potassium dichromate was firstly tested as a reference compound under these conditions. The number of the organisms is usually proportional to the volume of the test sample according to the literature (Vanhaecke *et al.,* 1981). Subsequently, four nauplii were inserted in each cell, which contained 4 ml of artificial seawater for controls and/or potassium dichromate solutions in various concentrations. The first results from the recorded videos

were encouraging but some further preliminary tests were also needed. In particular, a range of experiments had to be performed in order to verify that the behaviour of the nauplii in a cuvette was only affected by the presence of toxic compounds and not by natural changes in the behaviour of the test organisms. These experiments are detailed in Table 5.1.

At first, various temperatures were tested for identifying the range of temperatures for which the activity of the nauplii was substantially constant for 24 hours. Additionally, various levels of turbidity were applied to the solution in order to verify the tracking accuracy of the algorithm in variable solutions. Furthermore, by adding different numbers of nauplii in the toxic solutions, the relation between population size and the performance of the screening device was derived. In addition, many contaminants were analysed such as herbicides, pesticides, organic and inorganic compounds, mixtures of two contaminants (copper and cadmium ions) and wastewater samples for testing the broad application of the device to various contaminants (Table 5.2). Finally, the optimum exposure time of the nauplii to toxicants was identified by performing experiments after 16, 20 and 24 hours of exposure.

In the following sections, more details about the experiments performed and the results obtained will be analysed.

Table 5.1 Summary of the experiments perform to optimise the newly developed device.

Parameter	Treatment
Turbidity	10-90%
Temperature	0, 15, 25, 37, 50, 70° C
• Time	16, 20 and 24 hours
• Number of tested organisms	4 and 8 nauplii
Contaminants	**Cu^{2+}, Cd^{2+}, Fe^{2+}, Zn^{2+}, Phenol, Atrazine, Sodium hypochlorite**
• Time	16, 20 and 24 hours
• Number of tested organisms	4 and 8 nauplii
Contaminants	**Paraoxone, Colchicine, Aldicarb, Thallium sulphate**
• Time	24 hours
• Number of tested organisms	4 and 8 nauplii
Contaminants	**Cu^{2+}, Fe^{2+}, Zn^{2+}**
• Time	0 and 24 hours
• Number of tested organisms	"Many" nauplii

Table 5.2 Contaminants tested via this newly-developed device.

Category	Contaminant
Reference substance	Potassium dichromate
Metals	Cadmium, Copper, Iron and Zinc ions
Organic compound	Phenol
Inorganic compound	Sodium hypochlorite
Organophosphorous insecticide	Paraoxon-ethyl
Carbamate pesticide	Aldicarb
Pharmaceutical	Colchicine
Herbicide	atrazine
Rodenticide	Thallium sulphate
Mix of contaminants	Cadmium and Copper ions
Wastewater samples	Before, during and after ECP treatment
Total	**12**

5.1.5 Heavy metals in the aquatic environment

The aquatic environment is contaminated with heavy metals such as copper, cadmium, zinc, lead and mercury. Cadmium and lead have no known positive biological functionality. On the other side, copper and zinc for example, are necessary elements for the metabolism of organisms in low concentrations but in higher concentrations are toxic (Liu *et al.,* 2001).

Cadmium is a heavy metal, which was discovered in 1817, by German chemist, Friedrich Stromeyer. It is an important metal with many uses such as the electroplating for corrosion resistance, battery manufacture and as a colour pigment for plastics and paints (Nováková *et al.,* 2007; Liu *et al.,* 2001). Furthermore, cadmium also exists at high concentrations in phosphate fertilisers which usually results in soil and plant contamination (Nováková *et al.,* 2007). Cadmium has a high bioaccumulation capacity and has no particular biological function in live organisms (Svobodova *et al.,* 1993).

Copper exists in drinking water, food and beverages (Liu *et al.,* 2001). It is an essential element and is found also connected to enzymes which catalyse oxidation and reduction

reactions (Sikorska and Wolnicki, 2010). Copper metallo-enzymes are related to melanin production, collagen synthesis and cellular energy production (Lall, 2002). On the other side, high concentrations of copper in water threaten the aquatic organisms by producing endocrine disruption to fish (Liu *et al.,* 2001) and it is also utilised as an effective algicide (Murray-Gulde *et al.,* 2002).

Another metal that is abundant in the environment is iron. Iron is a biologically-essential metal and a major component of haemoglobin, mitochondrial enzymes, myoglobin, heme enzymes and metalloflavoprotein enzymes (Liu *et al.,* 2001). It usually exists in the forms of ferrous (+2) and ferric (+3) in biological systems. Accidental ingestion of iron-containing supplements constitutes the main reason for acute toxicity (Liu *et al.,* 2001). The symptoms are diarrhoea, vomiting and abdominal pain.

Zinc is also an essential metal and its deficiency has severe effects to health. It is present in water, air and food. The wide industrial use of zinc results in being one of the most common contaminant in water (Weiling *et al.,* 1991). In high concentrations zinc is toxic to organisms by preventing physiological activities (Weiling *et al.,* 1991). Additionally, it can be accumulated in organisms and transferred to higher food chains and even harm human health (Weiling *et al.,* 1991). The most common symptom is the "metal-fume fever" with symptoms of fever, cough, dyspnea, chest pains, muscle soreness, nausea, fatigue, chills and leukocytosis (Liu *et al.,* 2001).

Metal contamination in ecosystems can be caused by industrial processes and by tanks and pipes from local systems (Liu *et al.,* 2001). The metal toxicity differs depending on the environmental conditions and the aquatic species. For example, the hardness of water influences the chemical speciation of metals at a high degree.

In conclusion, the aforementioned metals have been widely studied for their toxic effect in various organisms according to the literature. In the following sections, the toxic effect of these metals to the mobility of *Artemia salina* nauplii will be extensively investigated using the newly-developed device. The results will be compared to the literature in order to validate the new system.

5.1.6 Study of the toxic effect of pesticides and pharmaceuticals on Artemia salina nauplii

Pesticides could be defined as "any substance or mixture of substances intended for preventing, destroying, repelling or mitigating pests" (Costa, 2001). Pests include weeds, rodents, insects and hosts of other organisms (Ecobichon, 2001b). There are four main categories of pesticides:

1. Insecticides (target group: insects)
2. Herbicides (target group: weeds)
3. Fungicides (target groups: fungi, moulds)
4. Rodenticide (target group: rodents)

However, there are also smaller categories of pesticides such as larvicides (larvae), molluscides (snails), acaricides (mites) and pediculocides (lice). Insecticides include carbamates, organophosphorous compounds, pyrethroids, organochlorines and other chemicals (Costa, 2001). Pesticides have been used commonly for many years in agricultural industry in order to control pests (Campanella *et al.,* 1996). As a result, they have been accumulated in the environment and have entered the food chain (Campanella *et al.,* 1996). Consequently, they are considered pollutants and the early detection of those in the water and in food is an imperative.

Aldicarb (2-methyl-2(methylthio)-propionaldehyde *O*-methylcarbamoyloxime) is a carbamate insecticide (Figure 5.1) which prevents the action of acetylcholinesterase (AChE) and subsequently the formation of choline. More specifically, AChE is an enzyme catalyses the transformation of acetylcholine (a neurotransmitter) into choline. If aldicarb exists, then acatylholine accumulates and does not allow the transition of nerve impulses across the conjunctions between nerves (Cox, 1992). As a result, loss of muscular coordination is caused which ends up to dizziness, sweating, nausea, vomiting as some of the effects to humans. In addition, aldicarb is applied in the form of granules underneath the soil surface. It is a systemic insecticide which means that the plant absorbs it from its roots and delivers it throughout the plant killing any insect exists on the flora.

Aldicarb belongs among the pesticides with the highest acute toxicity registered in UK and USA. The chemical characteristics of aldicarb such as the high mobility in soils and high solubility in water increase the potential of leaching to groundwater (Cox, 1992). Furthermore, toxic effects to humans from consumption of contaminated fruits and vegetables have also been reported. The human lethal dose concentration is 260 mg/l. Finally, aldicarb has been proved toxic to every species of life that has been exposed to it. Nowadays, the allowance of aldicarb use has been restricted. It can only be applied in approved areas which have low risk of leaching to groundwater (Buffin, 1999). Since many facts have been recorded, the need of early detection of contamination is a demand.
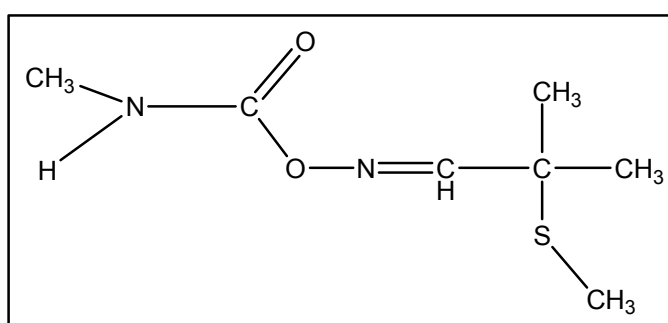
Figure 5.1 Chemical structure of Aldicarb.

Colchicine N-((7S)-5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo(a)- heptalen-7-yl)-acetamide is a pharmaceutical applied as a pain reliever and anti-inflammatory for people suffering from gout (Figure 5.2). It is also a highly poisonous alkaloid with human lethal dose of 240mg/l. It is originally extracted from plants and used to cure rheumatic complaints but it has also emetic and cathartic effects. Colchicine has been applied in order to cure certain types of solid tumours and leukaemia (Al-Mahmoud *et al.,* 2006).
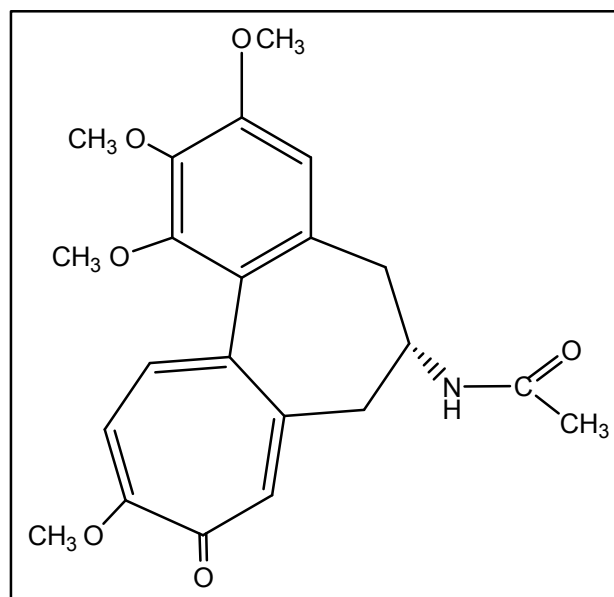
Figure 5.2 Structure of colchicine.

Thallium belongs to the heavy metals and naturally exists in the environment in low concentration (Kazantzis, 2000). Thallium is often found in sulphide ores of heavy metals such as lead, copper and zinc. Furthermore, in contaminated areas it can also be found in fruit, vegetables and farm animals at raised levels. Thallium also has industrial applications in the production of particular glasses, in electronics and in medical diagnostics. In addition, it is present in human tissues and is originated from vegetables with average intake of less than 5μg per day. Furthermore, thallium salts have been used as a poison since it is odourless, tasteless and colourless. However, due to rise in the number of poisoned animals (e.g. cats, dogs, foxes, martens) and its use for illegal abortions and homicidal purposes, it was banned. Thallium salts are categorised amongst the most toxic substances and applied in some Rodenticide. They can also be applied in the manufacture of chemical catalysts, imitation jewellery, optical lenses, low temperature thermometers and semiconductors. Regarding clinical applications, thallium isotopes are used in cardiac scanning and its salts used to treat scalp ringworm. The last treatment was abandoned due to the toxicity of the thallium salts. The selling of thallium in Britain is strictly licensed due to its use by murderers. In order the thallium to be considered toxic in humans, it should be accumulated in concentrations higher than 100 μg/l (Moore *et al.,* 1993).

Another substance used in the following experiments is paraoxon-ethyl (Figure 5.3). Paraoxon-ethyl is the active metabolite of the organophosphate insecticide parathion. Organophosphates have the same way of action as carbamates by inhibiting the action of

acetylcholinesterase. The difference between carbamates and organophosphates is that in the case of organophosphates the inhibition of acetylcholinesterase is not as easily reversible (Cox, 1992).
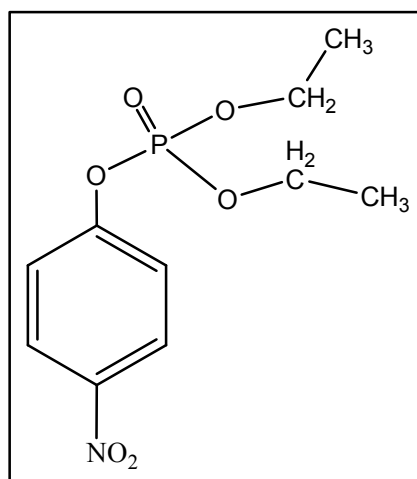
Figure 5.3 Structure of paraoxon-ethyl.

The previously discussed compounds, apart from paraoxon-ethyl, are the representatives of main toxicants categories proposed by Environmental Protection Agency (EPA) in order for innovative technologies to be verified (Environmental Technology Verification-ETV). This programme aims to better -protect the human health and the environment. Consequently, the performance of this newly developed device was tested with some of the proposed toxic compounds. In addition, paraoxon-ethyl tested for comparing the results with the already patented device by Portmann et al (1998), since paraoxon-ethyl was the tested compound used.

5.1.7 Combination of metals - Mixtures

The organisms in the natural environment are exposed to mixtures of contaminants at the same time. This exposure may lead to toxicological interactions. A toxicological interaction is defined as when an organism is exposed to two or more chemicals and its biological

response is different quantitatively or qualitatively than the expected one from the action of every chemical alone.

While two chemicals are provided to the tested organism simultaneously, their response could be additive or synergistic or potentiation or antagonistic. More specifically, an *additive* effect happens when the final effect of the two toxicants is equal to the total effects of every toxicant provided alone (e.g. 3 + 3 = 6). However, when the final impact of the two reagents is much higher than their sum when given alone then a *synergistic* effect has been occurred (e.g. 3 + 3 = 30). *Potentiation* happens when the one toxicant has not a toxic result to a specific system but when added in combination to another toxicant increases the toxicity of the second one (e.g. 0 + 3 = 10). Also, independent effects can be observed where the total impact of the contaminants is the same to the impact of the strongest pollutant alone. Finally, the *antagonistic* effect happens when the one chemical influence the other's action or they intervene to each other's actions (e.g. 3 + 0 = 1 or 3 + 5 = 6). Antagonistic effects of reagents consists the basis of some antidotes.

To sum up, the interaction happened among chemicals could be proved more or less toxic for the living organisms. However, this effect should be studied more since the toxicity of water does not always originate from only one substance (Rand *et al.,* 1995)

5.2   Materials and Methods

5.2.1 Mortality based assay

Preparation of test organisms

The *Artemia* cysts were purchased from *brineshrimpdirect.com* and were the species of *Artemia salina*. The hatching media used was artificial seawater (ASW) of 3.5 ± 1 % prepared with synthetic sea salt provided by Instant Ocean (Sarrebourg, France). The pH of the medium and of the tested samples should be 8.0 ± 0.5 otherwise it was adjusted with either hydrochloric acid or sodium hydroxide using a pH-meter, model HI8014, Hanna

instruments, (Leighton Buzzard, UK). 300ml of ASW and approximately 100mg of cysts were inserted in a 500ml conical flask and incubated under gentle aeration from an aquarium pump at $30 \pm 1^{o}C$. After 48 hours the nauplii was at the stage of instar II-III and ready to be used. Pictures were taken during the hatching period using a microscope (Zoom digital USB microscope, Vera Trinder Ltd., UK)

Reagents

The following reagents were used for the experiments and were of general laboratory grade unless stated otherwise:

Phenol was obtained from Sigma-Aldrich (Gillingham, UK). Hydrochloric acid, dimethylformamide (DMF) and copper nitrate $[Cu(NO_3)_2 \times 3H_2O]$ were provided by Fisher Scientific (Loughborough, UK). Sodium hydroxide was obtained from Acros Organics (Loughborough, UK). Potassium dichromate was provided by BDH (Lutterworth, UK), atrazine from Riedel de Haen (Hanover, Germany). The sodium hypochlorite used was from the commercially available bleach (Domestos, Uniliever, UK). Finally, Milli Q water was obtained from Millipore (Watford, UK).

Methodology

Artemia salina nauplii were grown in ASW (3.5%) until the stage of instar II-III (48-hour-old) and then were placed in toxic solutions for 24 hours. More specifically, 4.9 ml of ASW 3.5% were inserted to test tubes. Then, 0.1ml of distilled water (controls) or 0.1 ml of the toxicant at the appropriate concentration was added. At the end, 10 nauplii with the minimum volume of ASW carried over were inserted to the test tubes. After 24 hours at $25 \pm 1^{o}C$, the dead nauplii were counted and the LC_{50} values estimated with probit analysis. The nauplii were considered dead if no movement observed in 10 seconds (Vanhaecke *et al.,* 1981). Finally, the assay would be considered valid

1. if the percentage mortality of the control does not exceed the 10% (Vanhaecke et al., 1981) and

2. if the estimated LC_{50} for the reference substance (potassium dichromate) ranges between 30 and 50 mg/l (Svensson *et al.,* 2005).

The percentage mortality was calculated according to the following formulas. Firstly, the mortality factor was estimated from the mortality observed in the control samples (Equation 5.1):

$$\boldsymbol{mortality\ factor = \left[1 - \frac{N_{final}}{N_{initial}}\right]} \qquad (5.1)$$

Where $N_{initial}$ is the initial number of the inserted nauplii in the test tube and N_{final} is the number of the dead nauplii after 24 hours. If the mortality factor is lower than 1 (valid assay), then the percentage mortality will be calculated by multiplying the percentage mortality of each sample by the mortality factor (Equation 5.2).

$$\boldsymbol{\%\ mortality = \left[\frac{N_{final}}{N_{initial}} \times 100\right] \times mortality\ factor} \qquad (5.2)$$

5.2.2 Study of the lethal effect of various toxicants on *Artemia salina* nauplii

The tested compounds (a) atrazine, (b) copper ions, (c) phenol and (d) sodium hypochlorite were tested for their lethal effect on *Artemia salina* nauplii. Due to the low solubility of atrazine, DMF was used to facilitate its dilution. The methodology followed was described in the section 5.2.1.

The concentrations prepared were:

➢ 10^{-4}-10^{-8}M or 21.6-0.00216mg/l for atrazine,

➢ 10^{-1}-10^{-5} M or 24160-2.416mg/l for copper nitrate,

➢ 10^{0}-10^{-5} M or 94110-0.9411mg/l for phenol and

➢ 10^{0}-10^{-5} dilution for sodium hypochlorite

The dilutions were prepared with distilled water. Triplicates were performed for each dilution and for controls. The nauplii inserted to each sample were from the same generation and were not fed during the assay. After 24 hours of incubation, the dead nauplii were counted and the results were recorded. The 24-hour Lethal Concentration (LC_{50}) at which the 50% of the population died was calculated with Probit Analysis using Biostat 5.2.5.0 (AnalystSoft, 2008).

5.2.3 Mobility based assays

Preparation of test organisms

Artemia salina nauplii were hatched according to the process described in section 5.2.1.

Reagents

In addition to the reagents of the previous experiment, the following reagents were also used for the mobility based experiments and were of general laboratory grade unless stated otherwise:

Copper sulphate [$CuSO_4$ $5H_2O$], cadmium chloride [$CdCl_2$ xH_2O], colchicine, thallium (I) sulphate, paraoxon-ethyl (analytical standard) and aldicarb (analytical standard) were obtained from Sigma-Aldrich (Gillingham, UK). Zinc sulphate [$ZnSO_4$ $7H_2O$] and iron sulphate [$FeSO_4$ $7H_2O$] were obtained from Acros Organics (Loughborough, UK). Sea sand was also used which was obtained by Fisher Scientific (Loughborough, UK).

Description of the initial/preliminary device

The first design used for monitoring the nauplius movement consisted of a monocular camera (Canon Power shot S3 IS) placed on a tripod provided by Jessop's (Manchester, UK). 10ml round vials were placed on the top of the laboratory bench and videos were recorded for every sample.

Description of the improved design

The new designed device for monitoring the nauplii movement and consequently detecting the toxicity, consisted of the camera used in the above design, with its tripod, a dark chamber, LED torches (AAA battery torch, 7dayshop, Guernsey) and 4.5 ml polystyrene cuvettes provided by Fisher Scientific (Loughborough, UK). The camera was attached to the chamber with the LED illumination source underneath (Colour plate 5.4). The cuvettes containing the samples were placed inside the chamber in such a position that they could be properly illuminated by the light source (Colour plate 5.5). Videos were recorded for the controls and each sample for two minutes. A laptop computer running the newly-written algorithm for tracking the movement of the nauplii was used to analyse the videos. The digital image processing algorithm estimated the average speed of each shrimp by dividing the length of the tracking path by the recording time. So, the final value derived from the algorithm represents the average speed per nauplius in the sample. The Inhibitory Concentration (IC_{50}) represents the concentration of a contaminant at which 50 per cent of the mobility of the living organisms has been inhibited.

Colour plate 5.4 Dark chamber with attached camera and the light source underneath.
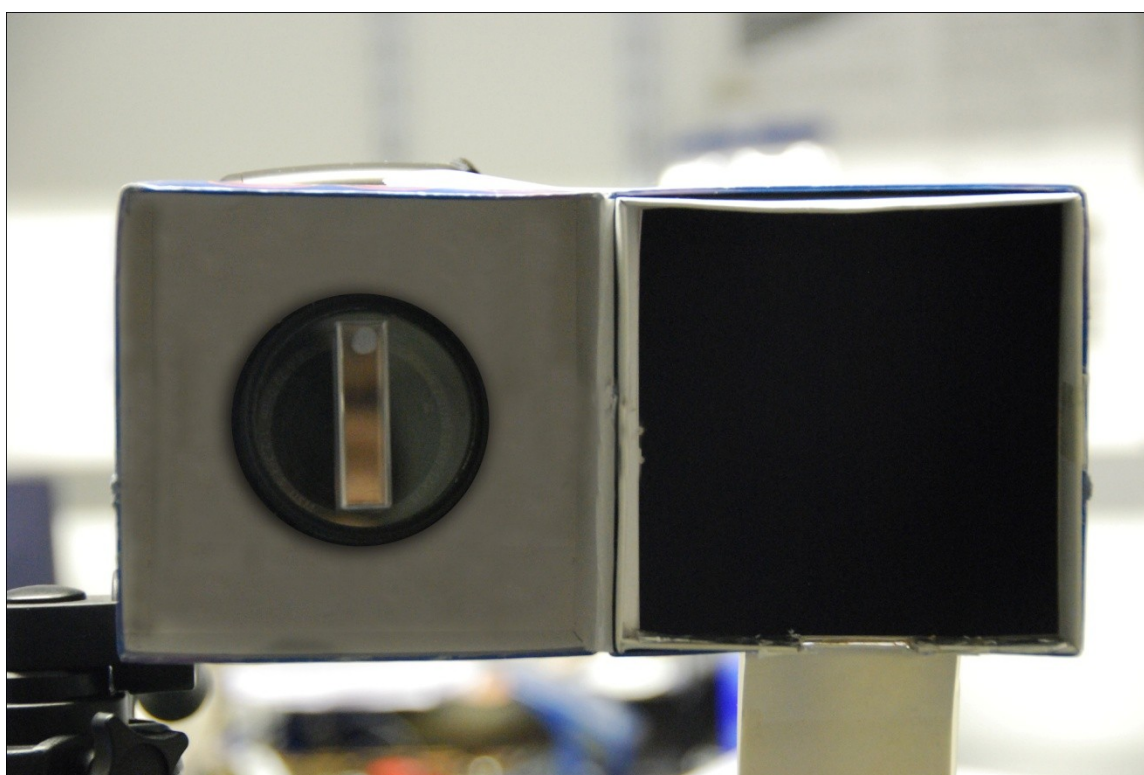
Colour plate 5.5 View of the inside of the dark chamber where. The cuvette was placed on the lens.

Methodology for the preliminary device

Preliminary assays were performed with the same compounds used for evaluating their lethal effect to *Artemia sali*na nauplii (Section 5.2.1) in order to validate the new device. The same dilutions of the contaminants were performed but in this case 10ml round vials were used. 5ml toxic solution was inserted to the vial with 10 nauplii and covered in order to prevent the evaporation and interference with the adjacent samples by dust effect. The incubation lasted for 24 hours at $25 \pm 1^{\circ}C$ and then videos were recorded for 20 seconds in open air environment. Triplicates were performed for each dilution and for controls. The nauplii inserted to each sample were from the same generation and were not fed during the assay. The videos were processed using the image processing algorithm and the results were further analysed. The measured mobility was the relative mobility since it was divided with the mobility of the controls. So, % ***Mobility*** and % ***Inhibition*** were calculated according to the following formulas (Equations 5.3 and 5.4):

$$\% \; \boldsymbol{mobility} = \frac{\boldsymbol{Vsample}}{\overline{\boldsymbol{V}} \; \boldsymbol{controls}} \times \mathbf{100} \tag{5.3}$$

$$\% \; \boldsymbol{Inhibition} = \left[\mathbf{1} - \frac{\boldsymbol{V \; sample}}{\overline{\boldsymbol{V}} \; \boldsymbol{controls}}\right] \times \mathbf{100} \tag{5.4}$$

Where V_{sample} represents the estimated speed per nauplius in the sample (m/s) as coming out from the algorithm and $\overline{V}_{controls}$ represents the average estimated speed per nauplius in the controls (m/s). IC_{50} was determined through the "best fit model" (Excel, Microsoft Office, 2007). The "best fit model" revealed that the most appropriate equation was the quadratic one.

Methodology for the improved design

The final procedure followed for analysing the samples, with the newly developed device, monitoring the mobility of *Artemia salina* nauplii was as follows:

The tested compounds were diluted in ASW in various concentrations. 4.5ml polystyrene cuvettes were used as test containers. 3.8 ml (unless stated otherwise) of ASW or each toxic substance was inserted in the cuvette with the appropriate number of tested organisms. Afterwards, the cuvettes were covered and after the appropriate period of incubation at 25 ± 1 $^{\circ}$C, the samples were monitored for two minutes and the videos were processed by the motion tracking algorithm. Many experiments were performed in order to optimise the procedure which will be described to the following sections.

% Mobility and % Inhibition were calculated according to the aforementioned equations 5.3 and 5.4 unless stated otherwise. Then, the results obtained were further analysed using statistical analysis (Section 5.2.11). The results are presented to the respective sections.

5.2.4 Preliminary experiments with the initial device

Atrazine, copper nitrate, phenol and sodium hypochlorite were firstly tested using the initial device. The dilutions were prepared in distilled water. Triplicates were performed for each dilution and for controls. The 10 ml round vials were filled with:

1. 4.9 ml of ASW (3.5%),
2. 0.1 ml of distilled water (controls) or toxic solution
3. 10 nauplii with the minimum volume of the hatching medium carried over.

Then, 20 second videos were recorded for all the samples. The videos were processed by the motion tracking algorithm and the results were further analysed.

5.2.5 Preliminary tests for optimising the final device

While implementing the improved design of the device for monitoring the mobility of the *Artemia salina* nauplii, some preliminary tests were also performed. The round vials were replaced with 4.5 ml polystyrene disposable cuvettes and the video duration was increased to 2 minutes. Videos of five control samples of ASW with 8 nauplii were initially recorded at $time_0$, when the nauplii were inserted to the cuvette, and after 24 hours for 2 minutes ($time_{24}$).

Afterwards, potassium dichromate was firstly tested as a reference compound. Four nauplii were inserted in each cuvette containing 4ml of potassium dichromate solutions at the concentrations of 9, 22.5, 45, 90 mg/l. The experiment was conducted in triplicate and the dilutions in ASW (3.5%).

Additionally, various temperatures were applied in order to specify the range where the nauplii retain their mobility for 24 hours. The tested temperatures were 15, 25, 37 and 50° C. The experiment was performed in triplicate for every temperature applied and videos were filmed at $time_0$ and after 3, 6, 9 and 24 hours. The percentage mobility of 8 nauplii in the aforementioned temperatures was estimated by comparing to controls of ASW and 8 nauplii from the same generation at laboratory temperature (25° C).

Another, optimisation experiment was to monitor the mobility of the nauplii in turbid solutions. Sea sand was used to prepare samples in gradually increasing levels of turbidity. Two 500ml beakers were used with 100 ml of ASW (3.5%). The respective amount of sea sand was added gradually in order to achieve increasing levels of turbidity. After the addition of the sea sand, 4ml of the mixture were inserted to the cuvettes. Then, the absorbance of each cuvette was estimated using the UV-Vis spectrophotometer (CamScan350). 8 nauplii were inserted to each cuvette and the experiment performed in duplicate. Controls of ASW with 8 nauplii were also filmed. Additionally, samples of each turbidity level were also recorded as control in order to minimise any potential error. Then, the average of the control values ($\overline{V}_{control}$, m/s) for each turbidity level was subtracted from the value of each sample with nauplii (V_{sample}, m/s) and the % mobility was estimated according to the following formula (Equation 5.5):

$$\% \ mobility = \frac{(Vsample - \overline{V}control)}{\overline{V}control} \times 100 \qquad (5.5)$$

Videos were processed by the motion tracking algorithm and the results were further analysed.

5.2.6 Comparison of the toxicity of metal salts using the mortality and mobility based assays

Before proceeding to more specific experiments and testing more contaminants and other parameters with the final device, an attempt to compare the lethal effect of toxic compounds to *Artemia salina* nauplii and the effect to their mobility was conducted. The LC_{50} and IC_{50} values were estimated and compared.

More specifically, potassium dichromate and cadmium, copper, iron and zinc salts were analysed. Stock solutions of 10^4 mg/l of all reagents were prepared in distilled water. Later, geometrically spaced concentrations were performed in ASW for each metal in quadruplicates with the exception of potassium dichromate where five replicates were performed. The pH of the samples was 8.0 ± 0.5. Four nauplii were inserted in 3.8 ml of ASW (controls) and in toxic samples. The dead larvae were counted after 24 hours of exposure and the LC_{50} values were evaluated with probit analysis. Two minute-videos were also recorded of the same samples and processed through the digital image processing algorithm. The results were further analysed using the equation 5.5 for higher sensitivity.

5.2.7 Study of the toxic effect of pesticides and pharmaceuticals on *Artemia salina* nauplii

The next step of the evolution of the developed device was to examine its sensitivity to more toxicants. Contaminants usually proposed by the Environmental Protection Agency (EPA)

for testing the sensitivity of new developed technologies for detecting toxic chemicals in water were tested by this device.

Stock solutions of 10^4 mg/l of aldicarb, colchicine, thallium sulphate and paraoxon-ethyl were prepared in distilled water. Then, geometrically spaced concentrations (10^3-10^{-4} mg/l) were conducted in ASW for each toxicant in triplicates. The pH of the samples was 8.0 ± 0.5. Subsequently, the same process as described in the methodology (Section 5.2.3) was followed. Two experiments were performed for each toxicant, one with 4 nauplii inserted to each sample and the other one with 8 nauplii. The dead nauplii were counted in each sample and afterwards it was recorded for 2 minutes in order the % inhibition to be estimated using the algorithm. Both experiments were conducted at $25 \pm 1\,^{\circ}$C.

Finally, the threshold toxicity which is defined as "t*he lowest concentration of contaminant to exhibit a percent inhibition significantly greater than the negative control*" (Schrock et *al.,* 2006) was estimated for each compound. In order threshold toxicity to be calculated, two significantly different inhibition values are required. When the standard deviation of the respective inhibition is added or subtracted to the inhibition value must not overlap the other concentration.

5.2.8 Study of the optimum exposure time of *Artemia salina* nauplii to the toxicants

Aiming to minimise the exposure time of *Artemia salina* nauplii to toxicants, videos of the samples were recorded after 16, 20 and 24 hours of exposure to the toxic compounds. The same procedure as described in section 5.2.2 at the *Methodology of the final device* was followed. Two experiments were performed for each toxicant, one with 4 nauplii inserted to each sample and the other one with 8 nauplii. Both experiments were conducted in quadruplicates at $25 \pm 1\,^{\circ}$C. The tested contaminants and the concentrations are presented in Table 5.3. The % inhibition was estimated for all the toxicants in each time interval for both experiments (4 and 8 nauplii).

Table 5.3 Tested contaminants for minimising the exposure time of *Artemia salina* nauplii to toxicants.

Contaminants	Concentrations (mg/l)
Cadmium ions	$10^3 - 10^{-4}$
Copper ions	$10^3 - 10^{-4}$
Ferrous ions	$10^3 - 10^{-4}$
Zinc ions	$10^3 - 10^{-4}$
Phenol	$10^3 - 10^{-4}$
Sodium hypochlorite*	$10^{-1} - 10^{-5}$

*measured as dilution, not absolute concentration

5.2.9 Development of a protocol using swarm of *Artemia salina* nauplii

An attempt to develop a screening device which could identify potentially toxic solutions was performed. Instead of inserting specific number of test organisms, which is a laborious task, a larger number of uncounted *Artemia salina* nauplii were inserted to each cuvette containing ASW only. Various procedures were applied in order to optimise a protocol.

Firstly, the process followed was to add the nauplii in a cuvette containing ASW, record the video for two minutes, remove the nauplii and finally insert them into the cuvette containing the toxic solution of the appropriate concentration. This was performed in order to record a control for each sample with the swarm of nauplii swimming in their natural environment. As a result, the comparison of their mobility in ASW and the toxic compound would be valid. However, this effort proved not effective and with high errors since the potential to lose some of the nauplii proved high. Consequently, no accurate results could be achieved. So, this process was not continued and the experiment conducted by adding the swarm of nauplii to 3.8ml of the toxic solution of copper, iron and zinc salts at the proper concentration and record the videos at time 0 ($time_0 = 0$ hours) and after 24 hours ($time_{24} = 24$ hours) (Methodology 1). Four replicates of each concentration for every compound were performed.

In order to improve the technique, it was decided to insert 3ml of ASW into each cuvette and swarm of nauplii with the minimum of ASW carried over. The nauplii were left for one minute to adjust themselves in the new environment before recording a 2 minute video

(time$_0$). Afterwards, the addition of 1ml of toxic solution of iron to the same cuvette was performed and finally the sample was ready to be incubated for 24 hours. The same procedure was applied for all the tested concentrations in five replicates. After 24 hours (time$_{24}$), the samples were recorded with the same order. Moreover, this procedure was conducted with four and an unquantified swarm of nauplii in iron sulphate solutions for comparison reasons (Methodology 2).

The % inhibition of the mobility of the nauplii was determined by dividing the mobility of the nauplii at time$_{24}$ by time$_0$. The formula used is the following:

$$\% \ Inhibition = \left[1 - \frac{V_{time24}}{V_{time0}}\right] \times 100 \qquad (5.6)$$

Where V_{time24} (m/s) is the speed per nauplius after 24 hours and the V_{time0} (m/s) is the speed per nauplius before the addition of the toxic sample at time$_0$.

The final optimisation was to add the swarm of nauplii in 4 ml of ASW instead of the 3ml used previously, record the video and then remove 1 ml of ASW and add the 1ml of toxic solution. This methodology was also abandoned after the first sample since the risk of removing some nauplii by accident was high. Subsequently, copper and cadmium salts were tested for their effects to swarm of nauplii using Methodology 2 in four replicates.

In conclusion, two main methodologies were followed and finally compared. The first one was to film the nauplii in the toxic solution at time$_0$ and time$_{24}$ and calculate the % inhibition from the equation 5.6. The second one was to record the swarm of nauplii in ASW for every sample at time$_0$, then add the toxic solution and after 24 hours record the samples again and estimate the % inhibition from the equation 5.6. Finally, controls with ASW were also recorded in all cases at time$_0$ and time$_{24}$. The whole experimental design is presented in Table 5.4.

Table 5.4 Experimental design.

Compound	Number of nauplii	Methodology	Concentrations tested (mg/l)
Copper	Swarm	1	10^{-4}-10^3
Iron	Swarm	1	10^{-4}-10^3
Zinc	Swarm	1	10^{-4}-10^3
Iron	4 nauplii	2	0.5,5,50,100,150,200,250,400,600
Iron	Swarm	2	0.5,5,50,100,150,200,250,400,600
Copper	Swarm	2	0.25,2.5,25,50,75,200,400,600,1000,1250
Cadmium	Swarm	2	0.0025,0.025,0.25,2.5,25,75,200,600,1000,1250

5.2.10 Study of the effect of combination of metals on the mobility of *Artemia salina* nauplii

Apart from testing individual contaminants for their toxic effect to *Artemia salina* nauplii, combination of cadmium and copper ions was also tested. Stock solutions of 10^4 mg/l of $CdCl_2$ x H_2O and $CuSO_4$ x $5H_2O$ were prepared in distilled water. Then, the second methodology of the previous section (5.2.9) was applied. The dilutions and mixtures performed are shown in the Table 5.5.

Then, the inhibition of the mobility caused was estimated using equation 5.6 and the type of the effect was identified (additive, synergistic, potentiation, antagonistic). The experiment was conducted in triplicate and a swarm of nauplii was used.

Table 5.5. Prepared concentrations of cadmium and copper ions and their mixtures.

Group	Concentration of CdCl$_2$ x H$_2$O (mg/l)	Concentration of CuSO$_4$ x 5H$_2$O (mg/l)
1	1250	0
2	1000	0
3	600	0
4	400	0
5	200	0
6	75	0
7	50	0
8	25	0
9	2.5	0
10	0	1250
11	0	1000
12	0	600
13	0	400
14	0	200
15	0	75
16	0	50
17	0	25
18	0	2.5
19	1250	1250
20	600	600
21	2.5	2.5
22	25	25
23	1250	2.5
24	2.5	1250
25	600	25
26	25	600
27	200	75
28	75	200

5.3 Results

5.3.1 Mortality based assay

The first approach performed using *Artemia salina* nauplii was the examination of the effect of various toxicants on their vitality. This assay constitutes a very common and quick assay the ARC- test (Artemia Reference Centre) (Vanhaecke *et al.,* 1981). The application of this assay leads to the calculation of the LC_{50} of the test toxicants. The determined LC_{50} values were utilised to compare the toxic effect of various contaminants on 48-hour old *Artemia salina* nauplii. The assessment of the toxic results of potassium dichromate, atrazine, copper ions, phenol, and sodium hypochlorite on the nauplii was initially tested and presented in the following section (5.3.2). Then, the LC_{50} estimation performed for all rest toxicants (cadmium, copper, iron and zinc salts and also Aldicarb, colchicine, paraoxon-ethyl and thallium sulphate) in order to compare the mortality and mobility assay.

5.3.2 Study of the lethal effect of various toxicants on *Artemia salina* nauplii

Potassium dichromate was initially applied as a reference substance. The LC_{50} value was 45mg/l which is in the valid range as proposed by Svensson *et al.* (2005). The mortality of *Artemia salina* nauplii in ASW (controls) ranged up to 10% for all the sets of experiments. The sensitivity range of the tested concentrations for each compound was between 241.60 and 24160 mg/l for copper, 9411 and 94110 mg/l for phenol and 10^0-10^{-5} dilution for sodium hypochlorite (Figure 5.4). Atrazine did not affect the vitality of the nauplii at the tested concentrations.
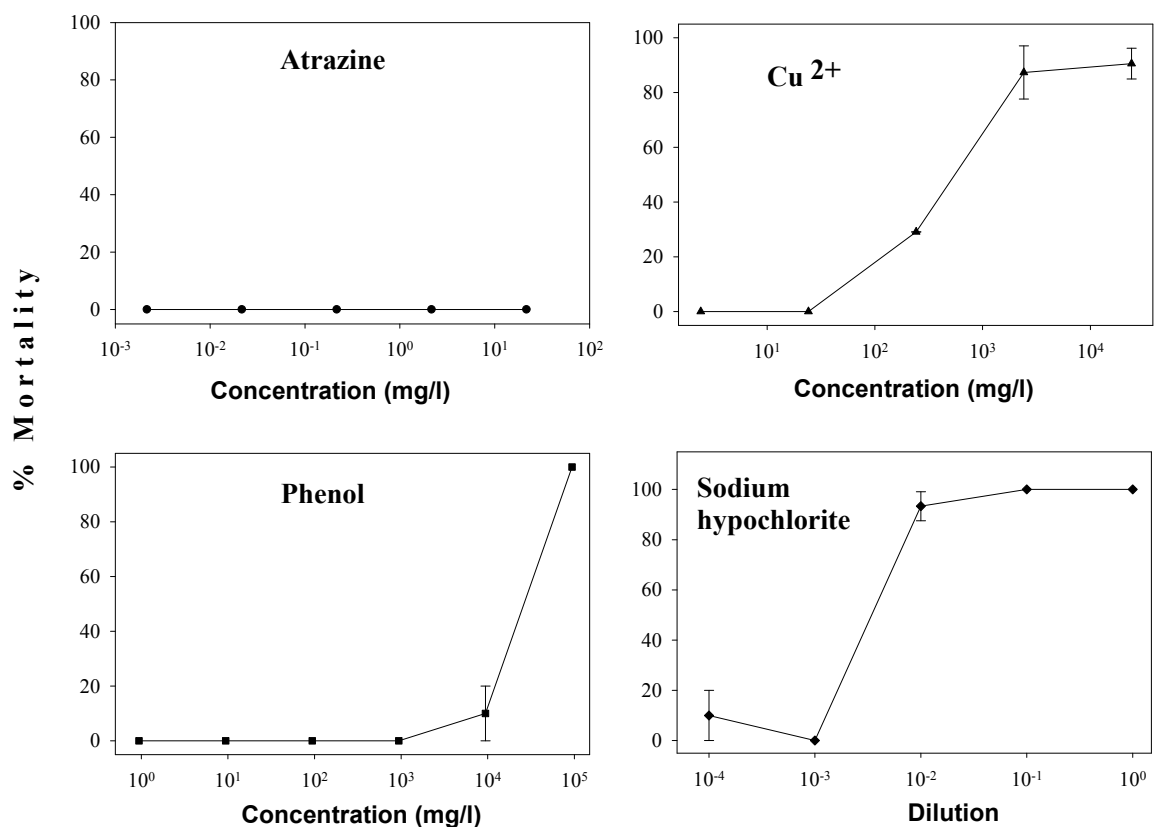
Figure 5.4 Percentage mortality curves for atrazine (●), copper ions (▲), phenol (■) and sodium hypochlorite (♦). The error bars represent the standard deviation of the mean of three replicates for each concentration (p<0.001).

The % mortality of *Artemia* nauplii followed a sigmoid pattern for copper ions and sodium hypochlorite with significantly different values as the concentration increased. Regarding phenol, the concentration range should have been expanded to more specific concentrations in order to reach a more specific trend. Apart from this, the % mortality of nauplii in phenol solutions showed a significantly different increasing pattern while the concentration also increased. Finally, the 24-hour LC_{50} were estimated for all the compounds using probit analysis (Table 5.6):

Table 5.6 LC$_{50}$ values of *Artemia salina* nauplii in the tested toxicants.

Substance	Calculated LC$_{50}$ (mg/l)
Potassium Dichromate	45
Copper ions	670.3
Phenol	16195.4
Sodium hypochlorite	207.8
Atrazine	-

5.3.3 Mobility based assay

Two generations of the newly-developed device, the initial and improved one, were applied in order to monitor the behaviour of *Artemia salina* nauplii in various toxic solutions and mixtures of those. Preliminary assays were initially conducted for both devices. Then, the mobility of the larvae was estimated under various conditions including, temperature and turbidity. Two different populations of nauplii were also applied for specifying the conditions under which the instrument was better performed. Furthermore, the results were compared to the mortality assay results in order to verify the findings obtained from the novel device for all the tested compounds. Additionally, the exposure time was minimised from 24 to 16 hours. Finally, a quick screening procedure was developed for assessing the potential toxicity of aqueous solutions using this device. The results of all the aforementioned experiments are described in detail in the following sections.

5.3.4 Preliminary experiments with the initial device

The mobility of the nauplii was monitored with the initial developed device. The results of each concentration were compared to the mobility of nauplii in ASW (controls). The same concentrations with the previous experiment (Section 5.3.2) were used for the tested toxic substances of atrazine, copper, phenol and sodium hypochlorite.

Firstly, according to Figure 5.5, the pattern showed that the nauplii were influenced by atrazine. Their mobility has been decreased but was approximately at the same level throughout the tested concentrations. At the highest concentration of 21.6mg/l, the mobility of the tested organisms was inhibited to 23.9% and at the lowest concentration of 2.16×10^{-3} mg/l to 19.50%.

In copper ion solutions, the results (Figure 5.5) showed that the percentage inhibition of the nauplii significantly increased as the concentration increased. The tested range was between 2.4 mg/l and 24160 mg/l and the corresponding inhibition was 27.8 and 88.12% respectively. The experimental pattern followed the expected dose-respond curve.

Then, the percentage inhibition of the mobility of the nauplii in phenol solutions was examined. The most diluted solutions did not provoke any inhibition to the mobility of the nauplii but it was significantly increased until reached 89.0% of inhibition of the mobility at 94110 mg/l. However, inconsistent values were recorded at 9.4110 mg/l which may be due to noise.

Regarding sodium hypochlorite, the percentage inhibition of the activity of the nauplii was significantly increased as the concentration became higher. The tested range was from 7.4 mg/l to 7440 mg/l with % inhibition values of mobility at 41.7% and 97.6% respectively.
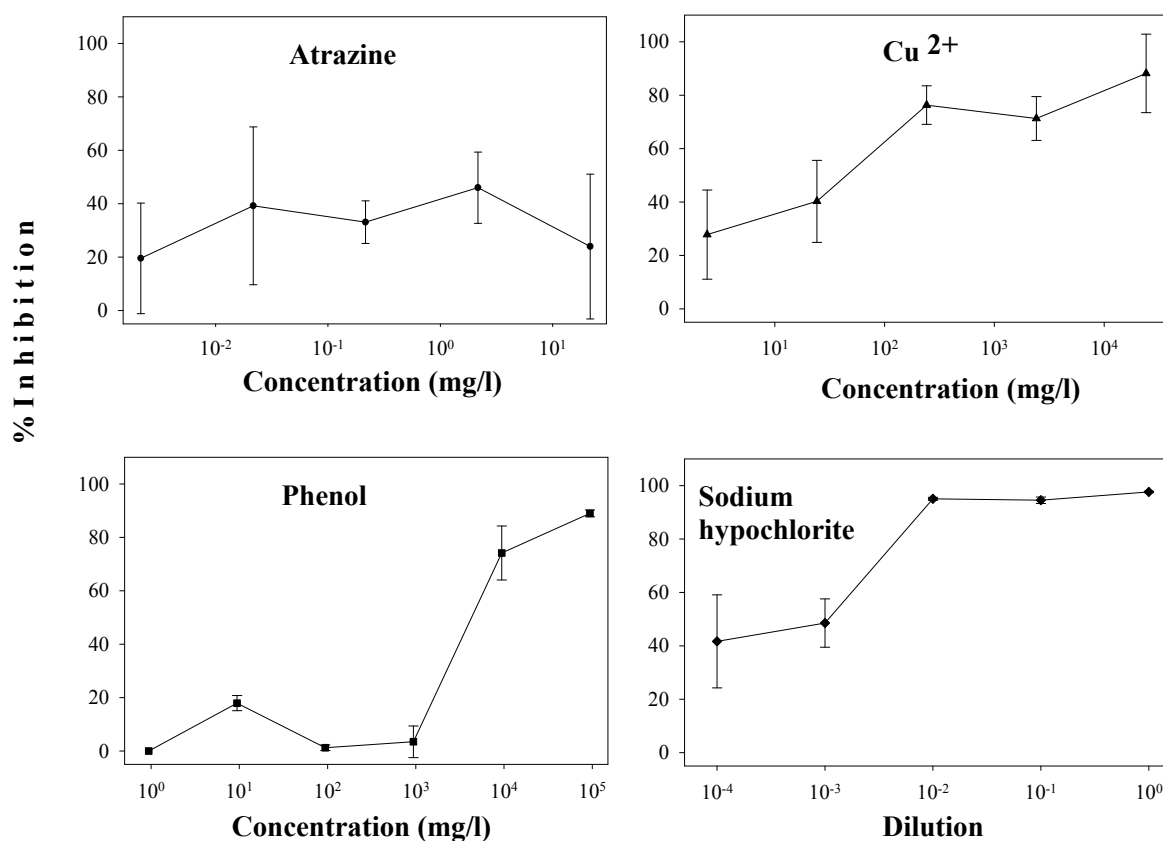
Figure 5.5 Percentage inhibition of mobility curves for atrazine (●), copper ions (▲), phenol (■) and sodium hypochlorite (◆) using the initial device. The error bars represent standard deviation of the mean of three replicates for each concentration with a $P < 0.001$ by analysis of variance (apart from atrazine).

5.3.5 Preliminary tests for optimising the final device

After improving the design of the device and optimising some conditions in the methodology, as analytically described in the section 5.2.3, the first experiment was to record samples with ASW and 8 nauplii at $time_0$ and $time_{24}$. The results from these videos showed that the average speed per nauplius of five replicates remained at the same levels for 24 hours (Table 5.7). As a result, the movement of the nauplii is not affected by time in controls. This experiment was repeated for every experiment and always the results verified that the mobility of the nauplii remained almost constant after 24 hours.

Table 5.7 Mobility of *Artemia salina* nauplii in ASW at $time_0$ and $time_{24}$.

Time (hours)	Speed (mm/s) per nauplius
0	11.444
24	11.636

Then the reference substance, potassium dichromate, was applied. The percentage mortality of the nauplii in the controls was 0% and the LC_{50} was slightly above the limits (30-50 mg/l) proposed by Svensson *et al.* (2005) and was 64.4 mg/l (Table 5.8). As observed from the graph (Figure 5.6), the % inhibition increased as the samples were more concentrated. The estimated IC_{50} was lower than the LC_{50} which is normal considering that the first one represents the concentration that inhibits the 50% of the mobility of the nauplii whereas the second one represents the concentration where the 50% of the nauplii are dead.

Table 5.8 Results from probit analysis and non-linear regression for potassium dichromate effect on *Artemia salina* nauplii.

Compound	LC_{50} (mg/l)	Fitted equation	R^2	Standard error	IC_{50} (mg/l)
Potassium Dichromate	64.4	$Y= -0.0049x^2+1.5982x-7.9779$	1	0.408	41.6
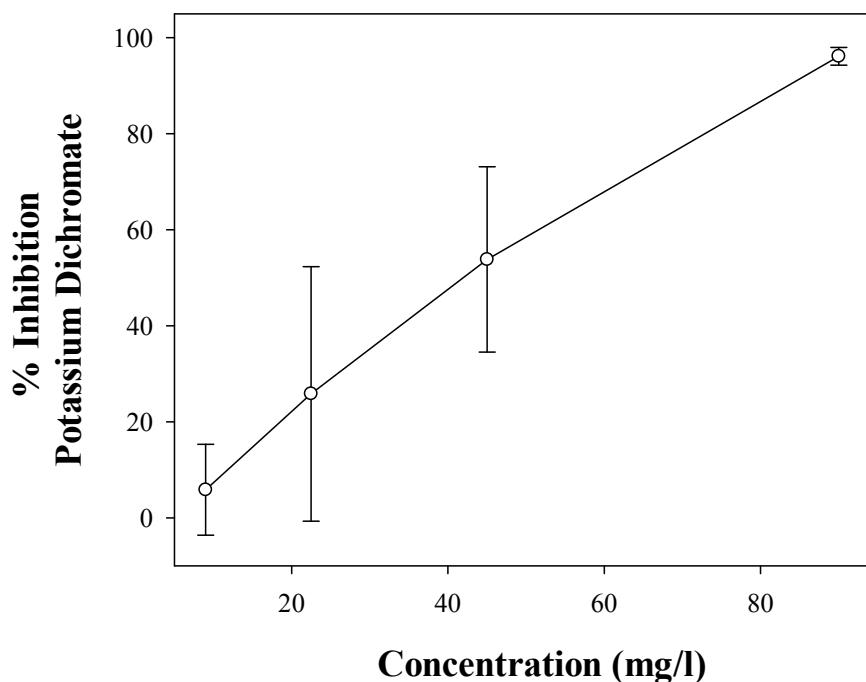
Figure 5.6 Percentage inhibition curve of mobility for potassium dichromate using the final device. The error bars represent standard deviation of the mean of three replicates for each concentration with a $P < 0.001$ by analysis of variance.

The next step was to ensure that the temperature did not affect mobility of the nauplii. So, the temperatures tested were 15, 25, 37 and 50° C with 8 nauplii in ASW (3.5%) and filmed at $time_0$ and after 3, 6, 9 and 24 hours. As shown in the following graph (Figure 5.7), at 15° C the % mobility of the nauplii was at high levels during the first 3 hours (98.2%) but then started to fall and reached 76.0% of their initial mobility. At 25° C the same levels of % mobility of the nauplii retained, around 90.0% for the first 9 hours, but after that the mobility dropped to 74.6%. Regarding 37° C, although the mobility of the tested nauplii increased after 3 hours compared to the initial one and remained higher for the rest of 6 hours, after 24 hours the mobility dropped to 64.5%. At 50° C, the percentage mobility of the nauplii increased from $time_0$ to 3 hours and then deteriorated to 18.8% after 24 hours of exposure. To sum up, the most suitable temperatures for testing the mobility of the nauplii ranged between 15° C and 25° C since the lowest drop of the mobility of the nauplii was observed.
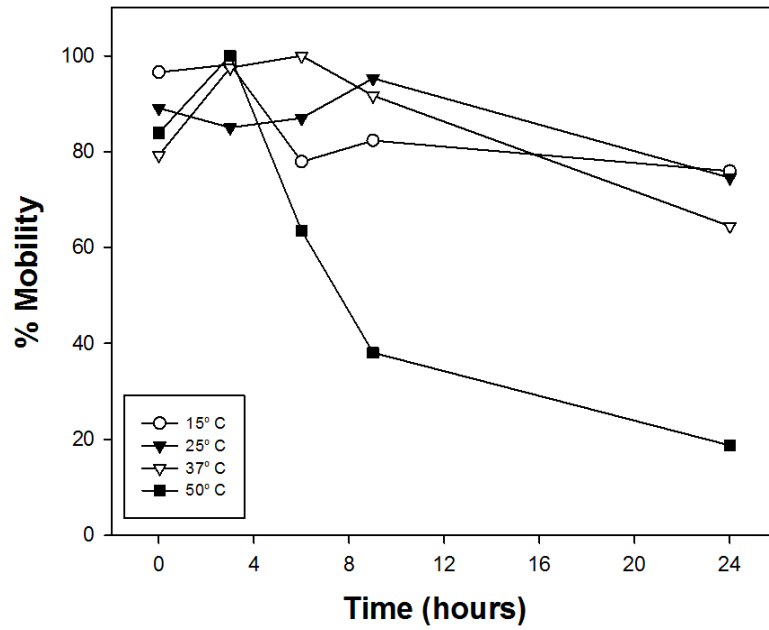
Figure 5.7 Percentage mobility of 8 nauplii in artificial seawater versus time at 15 (○), 25 (▼), 37 (▽) and 50° C (■). Mean of three replicates per temperature.

The last experiment conducted for optimising the performance of the device and the procedure followed was the application of various levels of turbidity (Colour plate 5.6).
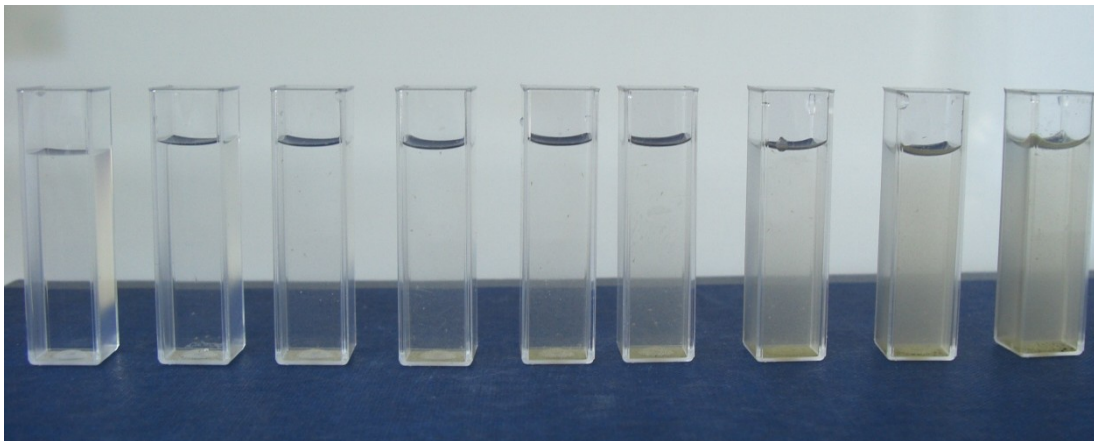
Colour plate 5.6 Gradual increase of turbidity levels from left (control) to right (most turbid sample).

According to the Figure 5.8, as the concentration of the sea sand increased, the levels of absorbance increased, as expected. A control with ASW with 8 nauplii was also recorded for each dilution and subtracted from the mobility of the sample as explained in the section 5.2.5 in order to minimise the noise. The results showed that the % mobility of the nauplii decreased as the concentration of the sand increased.
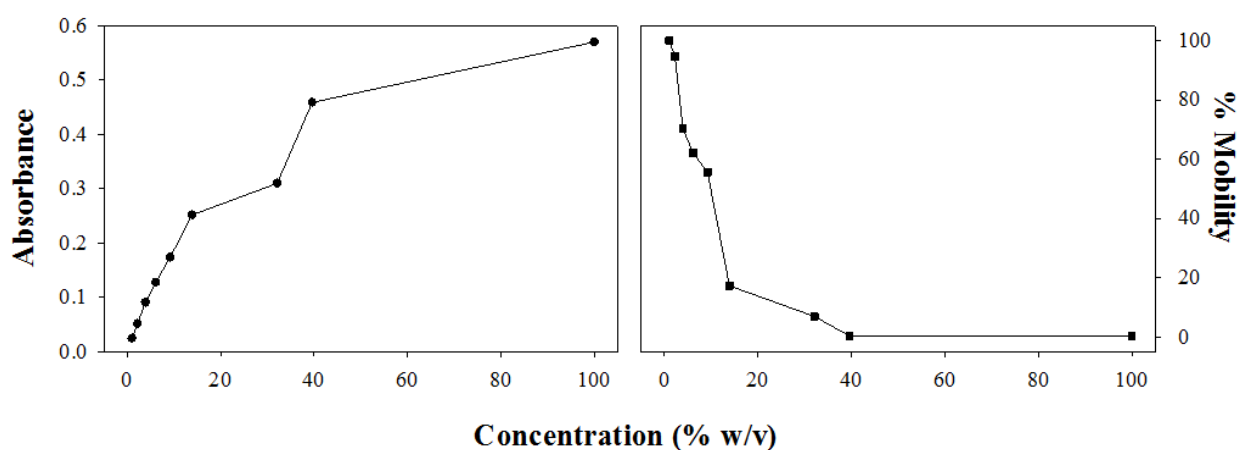
Figure 5.8 Absorbance and % mobility as estimated under various levels of turbidity with the UV-Vis spectrophotometer and the novel device. Mean of two replicates per treatment.

5.3.6 Comparison of the toxicity of metal salts using the mortality and mobility based assays

The effect of potassium dichromate on the inhibition of the mobility and mortality of *Artemia salina* nauplii were evaluated. The results were similar with the previous section with a slight increase of the values of LC_{50} and IC_{50} as presented in Table 5.9. In addition, the percentage inhibition curve had smaller error bars than the percentage mortality curve as shown in Figure 5.9. Additionally, from the inhibition curve more information from what happened to the tested samples can be derived. In particular, although the %mortality to the most concentrated sample of 100 mg/l was 70.0%, the percentage inhibition was 95.6%. This fact meant that some of the nauplii were alive but their mobility was limited. However, in both cases, the measured effect, percentage inhibition and mortality, significantly increased as the concentration became higher.

Table 5.9 LC$_{50}$ values (mg/l) as estimated using probit analysis and IC$_{50}$ (mg/l) values as estimated using the equations from the 'best fit model'.

Compound	LC$_{50}$ (mg/l)	IC$_{50}$ (mg/l)	Equation and R^2 from %inhibition graphs
Potassium dichromate	88.2	36.0	$y = -0.0122x^2 + 2.3922x - 20.295$ (R² = 0.9917)
Cadmium chloride	701.8	54.8	$y = -0.0008x^2 + 0.8407x + 6.2887$ R² = 0.9606
Copper sulphate	19.5	7.6	$y = -0.0366x^2 + 4.3648x + 18.721$ R² = 0.9683
Iron sulphate	NE*	66.3	$y = -0.0122x^2 + 2.3922x - 20.295$ R² = 0.9917
Zinc sulphate	1000	80.5	$y = -0.0003x^2 + 0.3933x + 20.294$ R² = 0.8493

*NE: Not Estimated
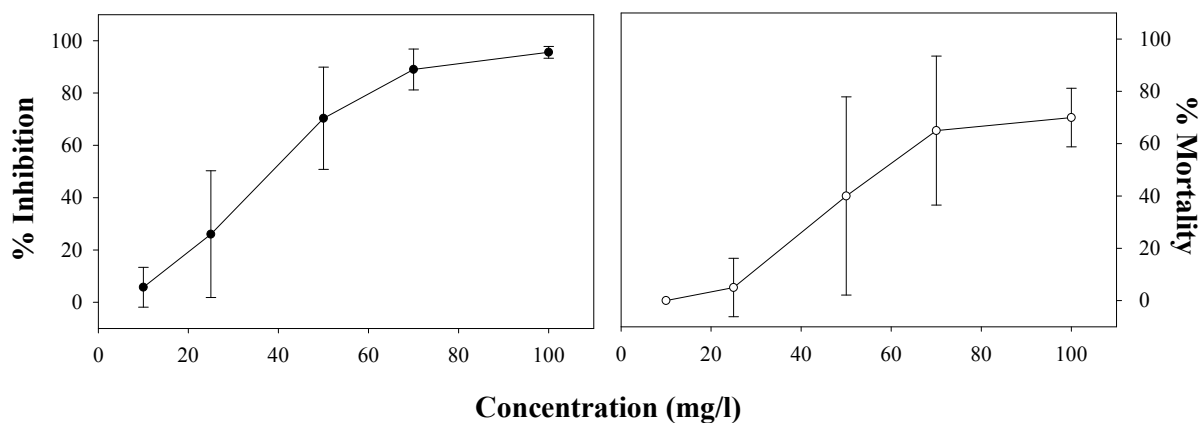
Figure 5.9 Percentage inhibition of mobility (●) and mortality (○) of potassium dichromate. The error bars represent the standard deviation from the mean values of five replicates for each concentration with a $P < 0.001$ by analysis of variance.

Furthermore, cadmium, copper, iron and zinc ions were analysed for their inhibitory effect of the mobility of the nauplii and the % mortality in order to compare the results (Figure 5.10)
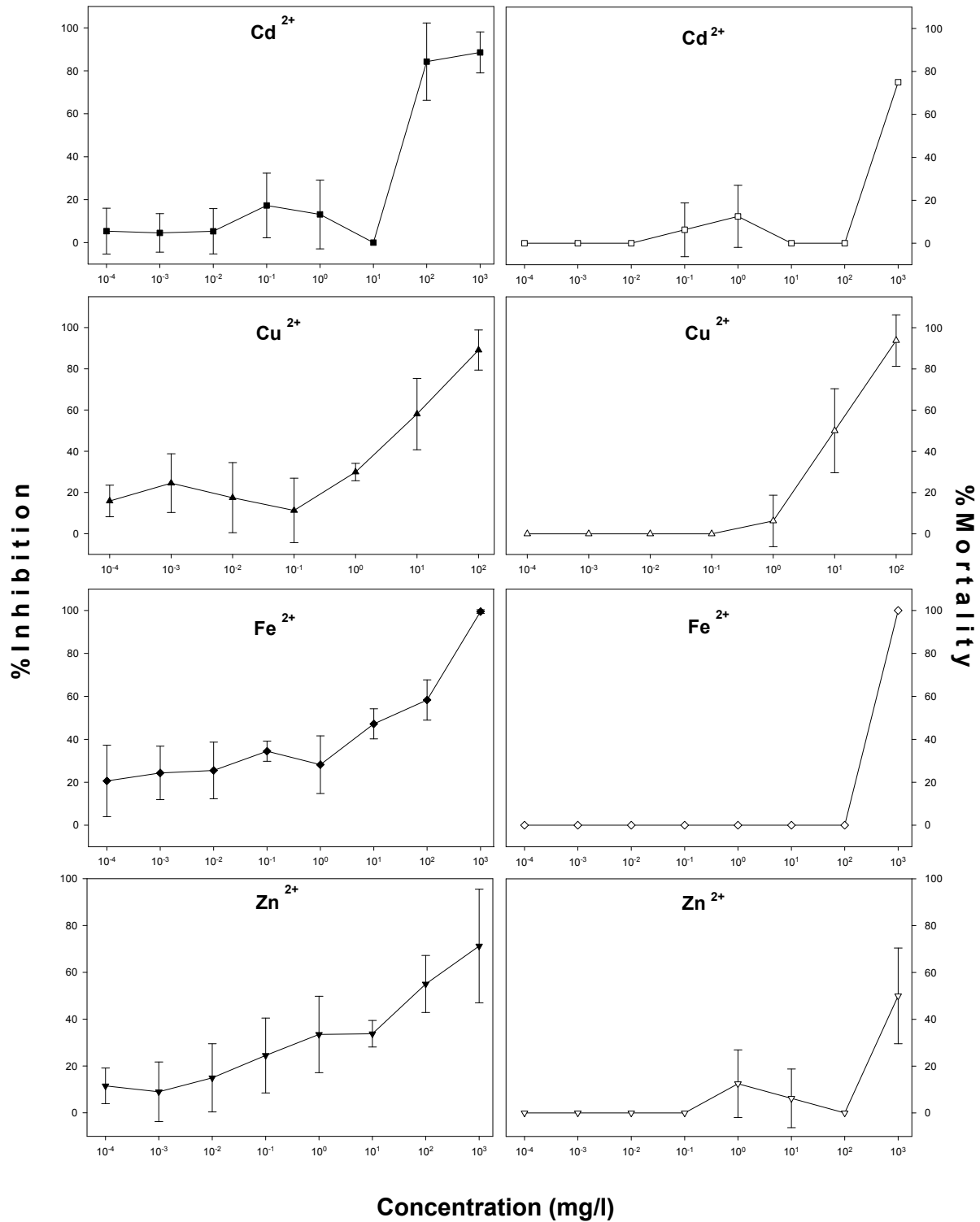
Figure 5.10 Percentage inhibition of mobility and mortality of *Artemia salina* nauplii in various metals: Cd^{2+} (■) and (□), Cu^{2+} (▲) and (△), Fe^{2+} (♦) and (◇) and Zn^{2+} (▼) and (▽) respectively. The error bars represent the standard deviation of the mean of four replicates for

each concentration with a P < 0.001 by analysis of variance apart from %mortality for iron ions which was not possible due to extreme results.

Regarding the recorded results for cadmium ions, both curves presented statistically significant effects. Although cadmium ions inhibited the mobility of the nauplii to 84.30% and 88.6% at 100 and 1000 mg/l respectively, the mortality effect was 75.0% at 1000 mg/l and 0% at 100 mg/l. The rest of the tested concentrations influenced neither the mobility of the nauplii nor their vitality.

The effect of copper to both examined effects showed that the sensitivity scale of %inhibition was shifted ten times more than the % mortality one. Particularly, the inhibitory effect was estimated to the extended range of 10^2 to 10^{-1} mg/l while the range for the lethal effect ranged between 10^2 and 10^0 mg/l. However, the estimated effects, inhibition and mortality, were significantly increased at the most concentrated samples.

According to the % inhibition and % mortality curves of iron ions, the sensitivity range was greater in the first case. Particularly, with the improved design of the device (final device), the effect of iron on the movement activity of the nauplii in the range of 10^3- 10^{-2} mg/l was measured. The more diluted samples (10^{-3} and 10^{-4} mg/l) inhibited at the same degree the mobility as occurred at the concentration of 10^{-2} mg/l. The growing effect of inhibition of the mobility of the nauplii to higher concentrations was statistically significant. On the other side, the % mortality was obvious only to the most concentrated sample at 1000 mg/l which provoke the 100% of the death of the tested nauplii. Due to these results, the LC_{50} value was not possible to be estimated. At the rest concentrations all the nauplii were alive. As a result, the final device showed higher sensitivity to identify the toxic effect of iron salts.
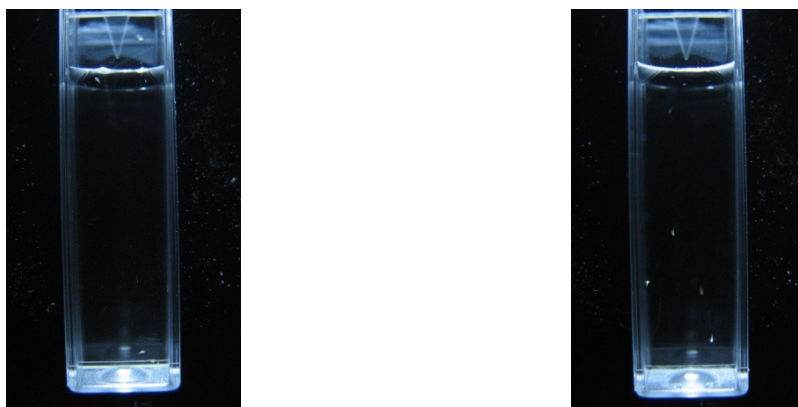
Colour plate 5.7 Toxic solution of Cd^{2+} in 10 mg/l and of Zn^{2+} in 100 mg/l.

Finally, zinc ions influenced the mobility of the nauplii among a great range of the applied concentrations, 10^3- 10^{-1} mg/l. In contrast, the % mortality was obvious only to the most concentrated sample of 10^3 mg/l. Moreover, *Artemia salina* nauplii were quite resistant according to mortality test since only the 50% of the population survived at the most concentrated sample. Nevertheless, both effects were significantly increasing to the highest tested concentrations.

In addition to these observations, the best fit model was also applied for the % inhibition curves in order to specify the equations representing the curves and the IC_{50} values to be calculated. The LC_{50} values for the examined metals obtained from probit analysis are also presented in Table 5.9. The IC_{50} values were lower than the LC_{50} ones meaning that with the mobility assay higher sensitivity was achieved. Furthermore, the toxic effect of metal salts can be identified in earlier stages than applying the mortality assay. Finally, in the Colour plate 5.4 the cuvettes containing cadmium and zinc are illustrated.

5.3.7 Study of the toxic effect of pesticides and pharmaceuticals on *Artemia salina* nauplii

The effect of aldicarb, colchicine, thallium sulphate and paraoxon-ethyl was tested on *Artemia salina* nauplii. The inhibition of the mobility and the lethal effect on the nauplii were evaluated. Two experiments were conducted for each endpoint, one with 4 nauplii per sample and one with 8 nauplii per sample in triplicates. The nauplii were all from the same generation. Controls with ASW and either 4 or 8 nauplii were also performed. The LC_{50} values were determined using probit analysis.

Figure 5.11 illustrates the significant increase of the percentage inhibition and percentage mortality as the concentration becomes higher for both experiments in aldicarb solutions. Using 4 or 8 nauplii per sample resulted to no significantly different results. However, the measurements of percentage inhibition using 8 nauplii had lower error comparing to the curve derived using 4 nauplii. In comparison to the inhibition curves, mortality results were less sensitive.

In more detail, the inhibition of the mobility was monitored with 4 nauplii inserted in the solutions from 1 mg/l to 1000 mg/l. The rest of the applied concentrations had almost the same impact on the reduction of the nauplii mobility. On the other side, the mortality effect on 4 nauplii was obvious only at 1000 mg/l considering that the value at 10 mg/l had high error.
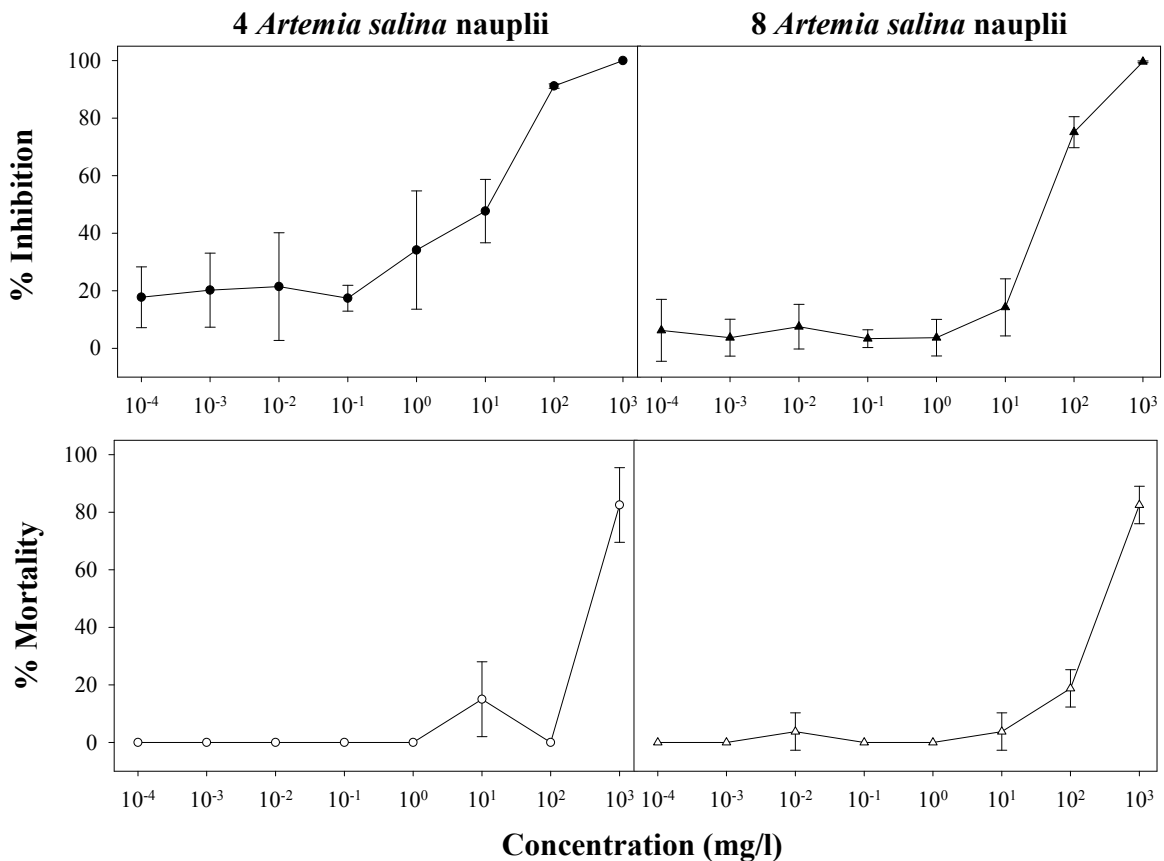
Figure 5.11 Percentage inhibition of mobility and mortality for aldicarb solutions for 4 nauplii (●) and (○) and for 8 nauplii (▲) and (△) respectively. The error bars represent the standard deviation of the mean of three replicates for each concentration with a $P < 0.001$ by analysis of variance.

The inhibition of mobility with 8 nauplii was monitored for a wide range of concentrations with the minimum of error. Particularly, from the concentration of 1mg/l until 1000mg/l the drop of the mobility of the nauplii could be monitored with high accuracy. In contrast, the mortality assay with 8 nauplii showed that the toxic effect of aldicarb could only be recorded for the concentrations of 100 mg/l and 1000 mg/l with percentage mortality of 18.7% and 82.5% respectively. However, the result at 10 mg/l had significant error. The rest of the tested

concentrations did not affect the vitality of the nauplii. Furthermore, the threshold toxicity was estimated at 10mg/l in both cases of 4 and 8 nauplii and presented in Table 5.11 at the end of this section.

The same pattern of the results was also followed while studying the colchicine effect on *Artemia salina* nauplii regarding the percentage inhibition curve (Figure 5.12). However, both the inhibitory effect and the mortality were significantly increased at the more concentrated samples. In addition, the sensitivity range in the case of inhibition was higher than the one of mortality for both experiments with 4 and 8 nauplii.

More specifically, the percentage inhibition with 4 nauplii could be evaluated from 0.1mg/l until 1000mg/l. The nauplii of the remaining tested concentrations retained the same mobility levels as occurred to the dilution of 0.1 mg/l. On the other side, the percentage mortality of 4 nauplii per sample ranged in low levels, 37.5 % and 22.5 %, and was only observed at the concentrations of 100 mg/l and 1000 mg/l respectively. The variance among the replicates as represented with the error bar was high and thus higher percentage mortality occurred to the less concentrated sample. The rest dilutions of colchicine did not provoke any lethal effect to the nauplii.

Regarding the results obtained with 8 nauplii per sample, the percentage inhibition could be determined between the concentrations of 1 mg/l and 1000 mg/l. The lower concentrations of colchicine did not significantly affect the mobility of the nauplii. As far as the % mortality of the nauplii in colchicine solutions, very inconsistent results were observed among all the tested concentrations.

The threshold toxicity was also evaluated from the percentage inhibition results and was 1 mg/l and 10 mg/l when 4 nauplii and 8 nauplii applied per sample respectively (Table 5.11).
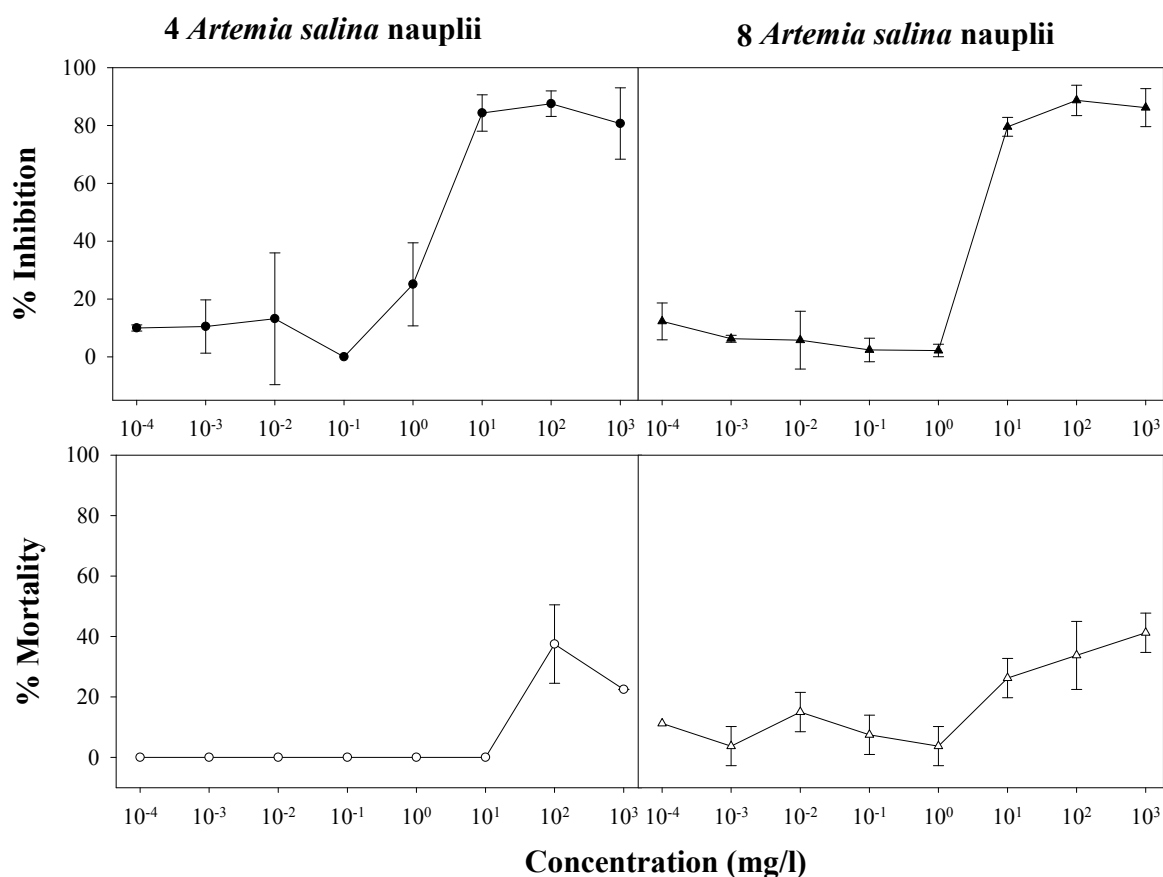
Figure 5.12 Percentage inhibition of mobility and mortality for colchicine solutions for 4 nauplii (●) and (○) and for 8 nauplii (▲) and (△) respectively. The error bars represent the standard deviation of the mean of three replicates for each concentration with a P < 0.001 by analysis of variance.

The inhibition of the mobility of the nauplii and the % mortality provoked by thallium sulphate was significantly increased as the samples became more concentrated (Figure 5.13). More specifically, from the concentration of 1 mg/l until 1000 mg/l the mobility of 4 nauplii was inhibited 8.0% and 100.0% respectively. The measurements of the least concentrated samples were very inconsistent. In comparison, the impact of thallium sulphate to the vitality of the nauplii in the same samples was obvious at the concentrations of 10 mg/l to 1000 mg/l with correspondent values of 15.0% and 90.0%. The more diluted samples did not influence the vitality of the nauplii at any degree.

Regarding the experiment with 8 nauplii per sample, the inhibitory effect of the mobility was easily distinguished between the concentrations of 10 mg/l and 1000 mg/l, where 22.7% and 100% inhibition were observed, respectively. The percentage mortality was only estimated

between the concentrations of 100 and 1000 mg/l with respective values of 18.7 and 71.2%. Although all the nauplii remained alive, the mobility of the nauplii was inhibited at 10 mg/l.

The sensitivity, when measured via the %inhibition, was higher than when the mortality assay was used, irrespective of the number of nauplii. In addition, the value of the threshold toxicity was ten times lower when 4 nauplii were applied to the samples, although larger error bars resulted (Table 5.11).
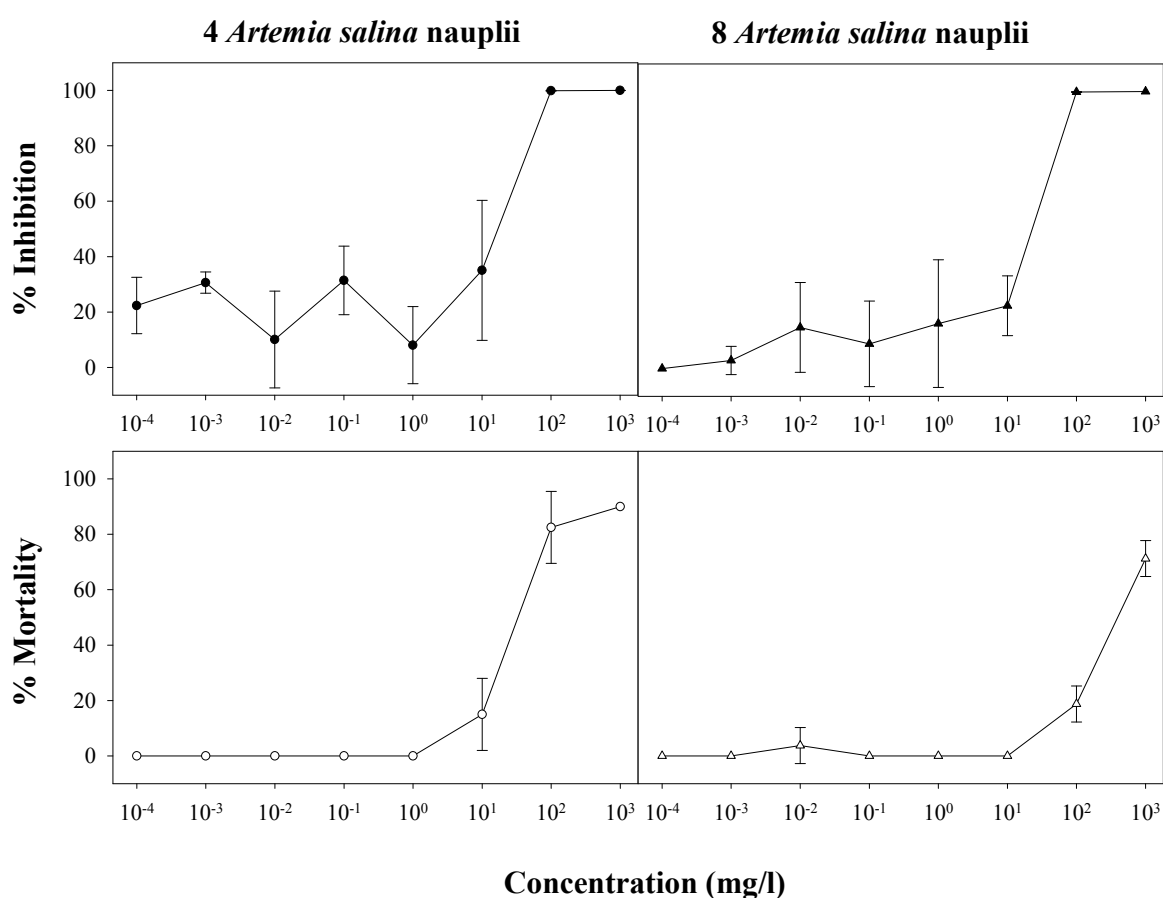
Figure 5.13 Percentage inhibition of mobility and mortality for thallium sulphate solutions for 4 nauplii (●) and (○) and for 8 nauplii (▲) and (△) respectively. The error bars represent the standard deviation of the mean of three replicates for each concentration with a P < 0.001 by analysis of variance.

Paraoxon-ethyl significantly increased the inhibitory effect on the mobility of the nauplii and likewise the lethal impact in both experiments, with 4 and 8 nauplii, as the samples became more concentrated (Figure 5.14).

The growing inhibition of the mobility of 4 nauplii was observed at the concentrations of 1 mg/l to 1000 mg/l with corresponded values of 11.4% and 100.0%. The rest tested concentrations influenced the mobility of 4 nauplii at the same degree as 1 mg/l. The results from the mortality effect did not follow the normal pattern and were inconsistently changed.
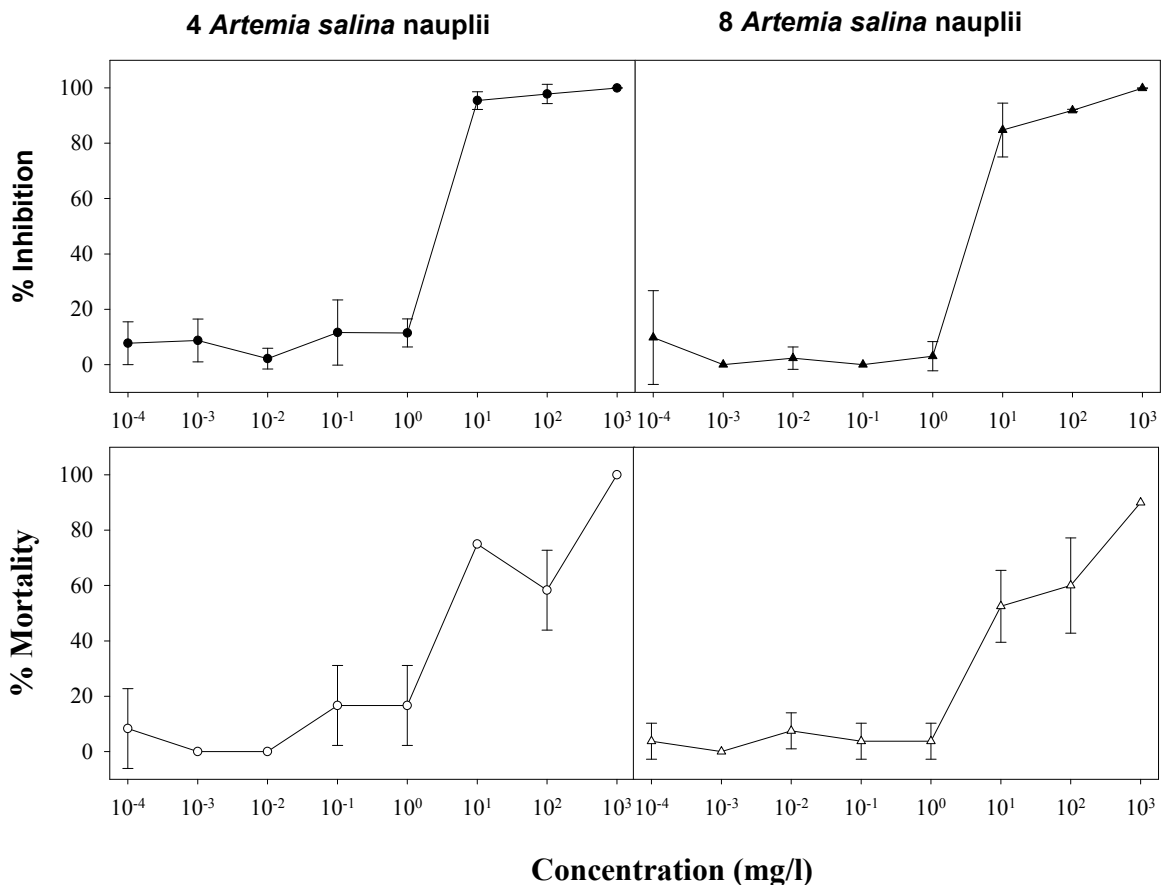
Figure 5.14 Percentage inhibition of mobility and mortality for paraoxon-ethyl solutions for 4 nauplii (●) and (○) and for 8 nauplii (▲) and (△) respectively. The error bars represent the standard deviation of the mean of three replicates for each concentration with a P < 0.001 by analysis of variance.

The critical range of the action of paraoxon-ethyl to the mobility of 8 nauplii was the same as with 4 nauplii (1-1000 mg/l). However, the % mortality affected in the same manner the vitality of the nauplii as monitoring the inhibition of the mobility. In addition, the threshold toxicity values were 10 mg/l for both experiments (Table 5.11).

Table 5.10 Equations for the percentage inhibition curves using the 'best fit model'.

Compound	Equations from % Inhibition graphs and R^2 (4 nauplii)	Equations from % Inhibition graphs and R^2 (8 nauplii)
Aldicarb	$y = -0.0007x^2 + 0.7493x + 24.745$ ($R^2 = 0.9428$)	$y = -0.0007x^2 + 0.7698x + 5.0008$ ($R^2 = 0.9981$)
Colchicine	$y = -0.0008x^2 + 0.8712x + 15.477$ ($R^2 = 0.6894$)	$y = -0.0007x^2 + 0.7959x + 21.36$ ($R^2 = 0.6217$)
Thallium sulphate	$y = -0.0008x^2 + 0.8695x + 21.26$ ($R^2 = 0.9434$)	$y = -0.0009x^2 + 1.0042x + 9.007$ ($R^2 = 0.9833$)
Paraoxon-ethyl	$y = -0.0009x^2 + 0.9358x + 19.945$ ($R^2 = 0.6555$)	$y = -0.0008x^2 + 0.9307x + 13.849$ ($R^2 = 0.6999$)

Regression coefficients for colchicine and paraoxon-ethyl indicate poor correlation with the best-fit curves from other experiments (section 5.4.3).

Table 5.11 LC_{50} (mg/l) and IC_{50} values (mg/l) as estimated from the mortality and mobility assays and also the values of threshold toxicity (mg/l).

Compound	LC_{50} (mg/l) 4 nauplii	LC_{50} (mg/l) 8 nauplii	IC_{50} (mg/l) 4 nauplii	IC_{50} (mg/l) 8 nauplii	Threshold (mg/l) 4 nauplii	Threshold (mg/l) 8 nauplii
Aldicarb	716.8	316.6	34.8	61.9	10	10
Colchicine	4,589.0	71,385.4	37.2	41.2	10	10
Thallium sulphate	70.87	428.6	34.1	42.4	100	100
Paraoxon-ethyl	19.1	24.4	33.2	40.2	10	10

In conclusion, the number of the tested nauplii did not provoke any statistically significant difference among the results obtained in all cases regarding both the inhibition and the

mortality. The IC_{50} values were estimated from the equations derived from the 'best fit model' (Table 5.10). The LC_{50} values presented in the Table 5.11 were relatively high comparing to IC_{50} values with the exception of paraoxon-ethyl. Paraoxon-ethyl had lower values for the mortality assay. Finally, in all cases the IC_{50} values obtained with 4 nauplii were lower than the ones with 8 nauplii.

5.3.8 Study of optimum exposure time of *Artemia salina* nauplii to the toxicants

Six toxicants were applied in order to monitor their inhibitory effect after 16, 20 and 24 hours on 4 and 8 *Artemia salina* nauplii. In general, the effect of the tested compounds was analysed and three-way analysis of variance (ANOVA) was performed (Appendix A, Tables A-51 – A-56) since 3 variables exist in these experiments: Concentration, Number of the nauplii and Time. The null hypothesis was that none of the variables affect the inhibition of the mobility of the nauplii.

According to Figure 5.15, in all graphs the % inhibition provoked by cadmium ions significantly increased as the concentration of the samples increased. Furthermore, the variance among four replicates was higher comparing the error bars for 4 nauplii than for 8 nauplii. However, according to the ANOVA table (Appendix A, Table A-51) the P-value, labelled ".F pr", the interaction among 'Concentration', 'Nauplii number' and 'Time' had P-values higher than the significance level. Subsequently, the null hypothesis was accepted that the 4 and 8 nauplii responded in the same way to different time intervals and concentrations in the inhibition of the mobility of the nauplii. To sum up, the number of the nauplii in the tested samples and the various exposure times did not affect the mobility of the nauplii.
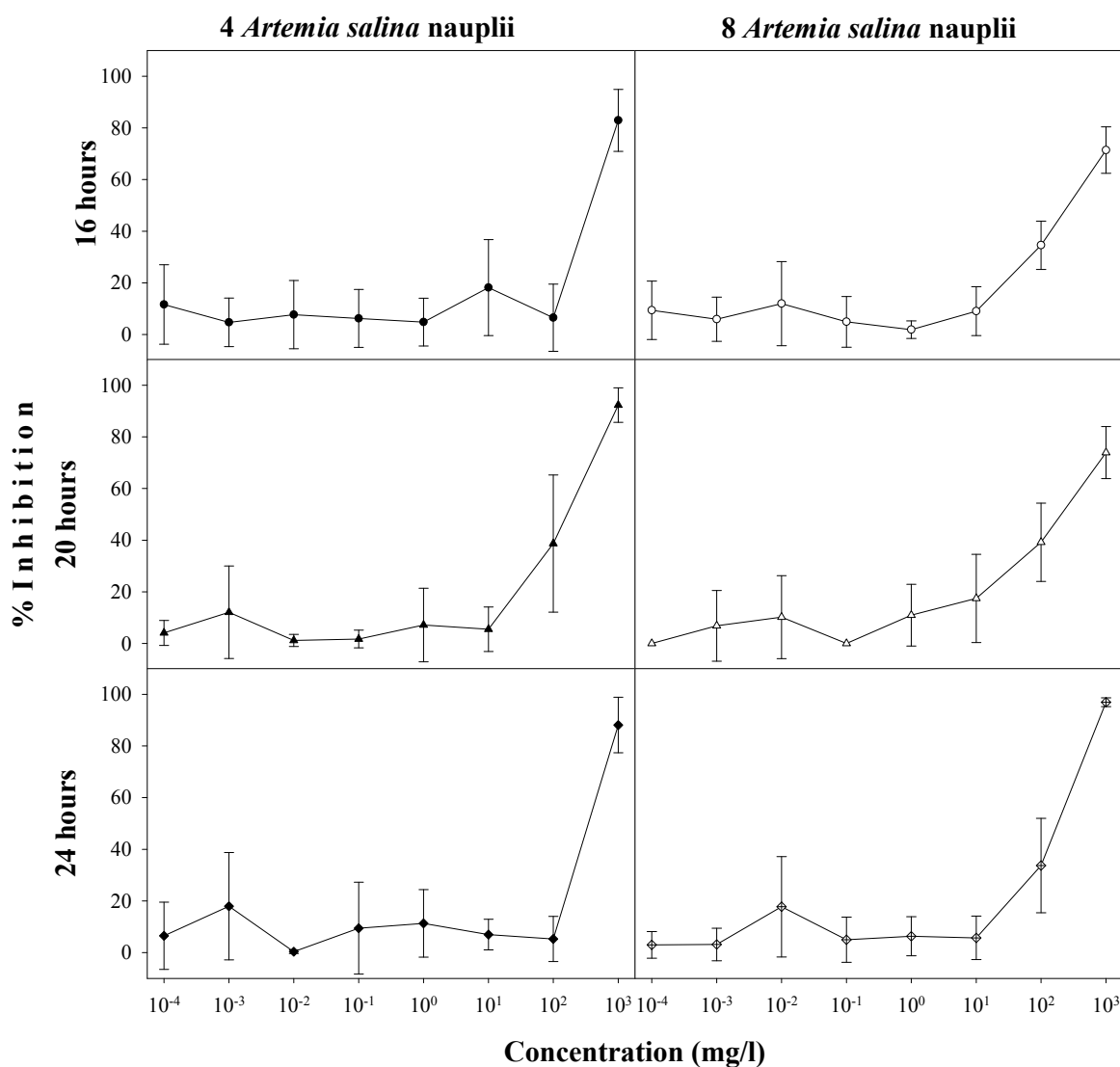
Figure 5.15 Percentage inhibition of the mobility of the nauplii after 16 (●) and (○), 20 (▲) and (△) and 24 hours (◆) and (◊) in cadmium ion solutions with 4 nauplii (black symbols) and 8 nauplii (white symbols) respectively. The error bars represent the standard deviation of the mean of four replicates for each concentration.

The same analysis performed for copper as for cadmium ion samples. The percentage inhibition of the mobility of the nauplii was significantly increased at the more concentrated samples according to Figure 5.16. The variance among the replicates was relatively higher when 4 nauplii applied in comparison to the respective effects when 8 nauplii inserted into the tested samples. The 24-hour curve for 4 nauplii was inexplicably inconsistent. This is one of the few examples where the device failed due to the low number of the nauplii. Regarding the ANOVA analysis performed (Appendix A, Table A-52), the interaction of

'Concentration', 'Nauplii number' and 'Time' had p-value higher than 0.05 so the null hypothesis was accepted. This means that both populations of nauplii (4 and 8) responded in the same way in various concentrations of the toxic compound and at different exposure times in the inhibition of their mobility.
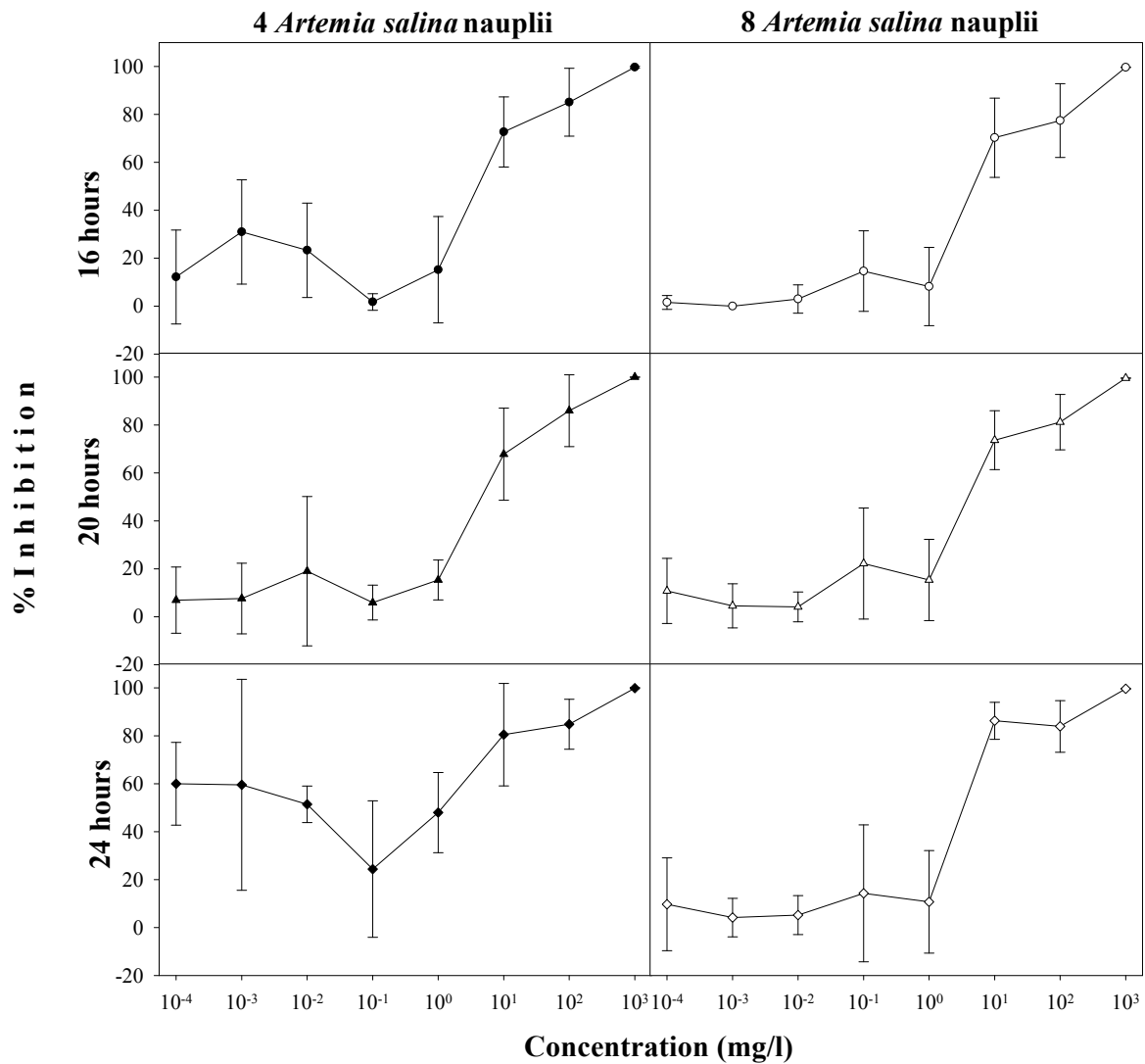
Figure 5.16 Percentage inhibition of mobility of the nauplii after 16 (●) and (○), 20 (▲) and (△) and 24 hours (◆) and (◊) in copper ion solutions with 4 nauplii (black symbols) and 8 nauplii (white symbols) respectively. The error bars represent the standard deviation of the mean of four replicates for each concentration.

Regarding the inhibitory effect of ferrous ion dilutions on the mobility of the nauplii, significant increase was observed in the more concentrated samples according to Figure 5.17.

The variance among the replicates was relatively higher in some cases when 4 nauplii applied than the respective effects when 8 nauplii used. According to the ANOVA analysis (Appendix A, Table A-53), the interaction among 'Concentration', 'Nauplii number' and 'Time' had not statistically significant value so the null hypothesis was accepted. This means that both populations of nauplii (4 and 8 nauplii) were affected at the same degree when this wide range of concentrations of the toxic compound applied and at different exposure times.
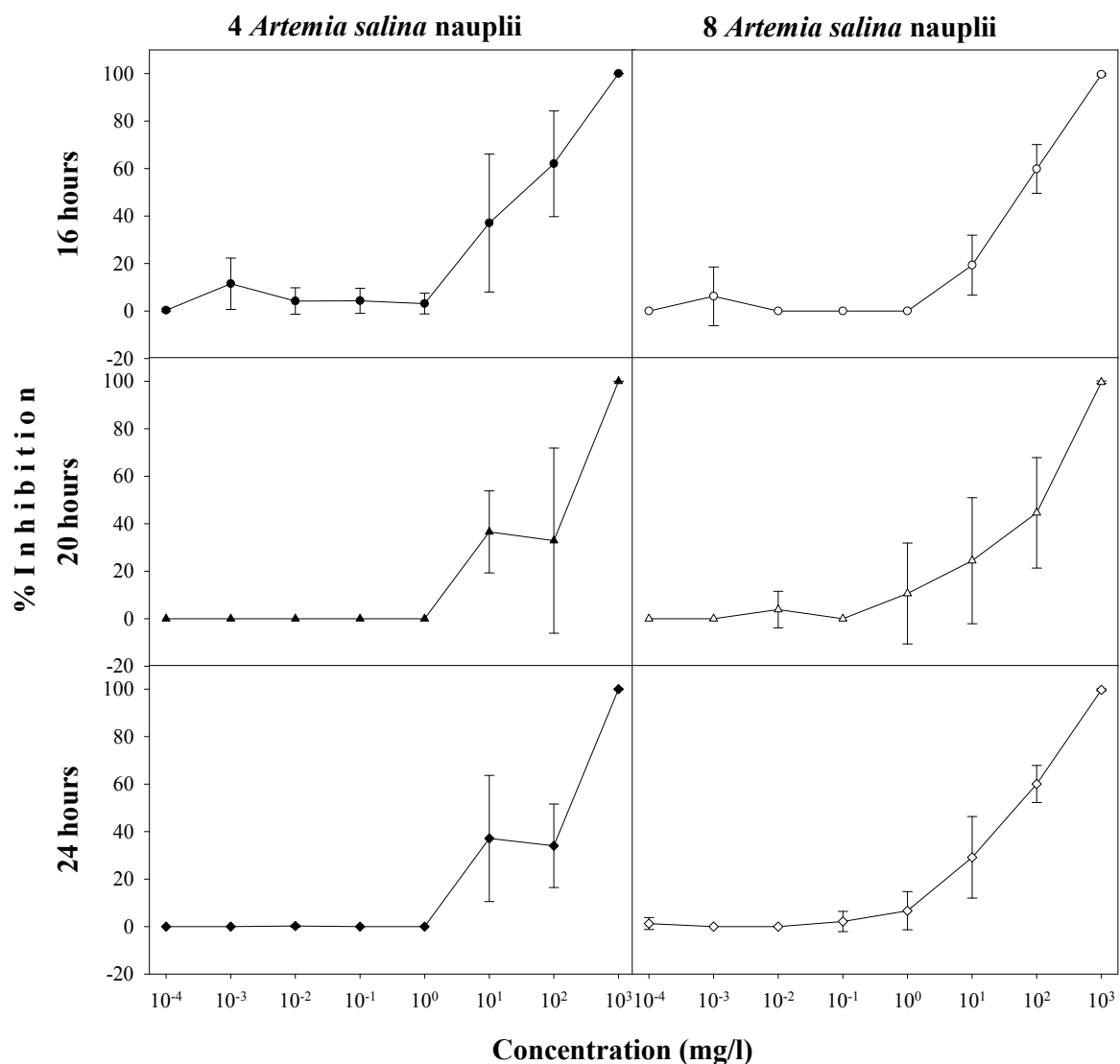
Figure 5.17 Percentage inhibition of the mobility of the nauplii after 16 (●) and (○), 20 (▲) and (△) and 24 hours (♦) and (◊) in iron ion solutions with 4 nauplii (black symbols) and 8 nauplii (white symbols) respectively. The error bars represent the standard deviation of the mean of four replicates for each concentration.

As far as the effect of zinc ions on the percentage inhibition of the mobility of the nauplii, it was significantly increased as the concentration became higher (Figure 5.18). Regarding the ANOVA analysis performed (Appendix A, Table A-54) the interaction of 'Concentration', 'Nauplii number' and 'Time' was higher than 0.05. Consequently, the null hypothesis was accepted. Both populations of nauplii (4 and 8 nauplii) were affected in the same way from the tested concentrations of the toxic compound. Furthermore, different exposure periods did not influence the inhibition of the nauplii mobility.
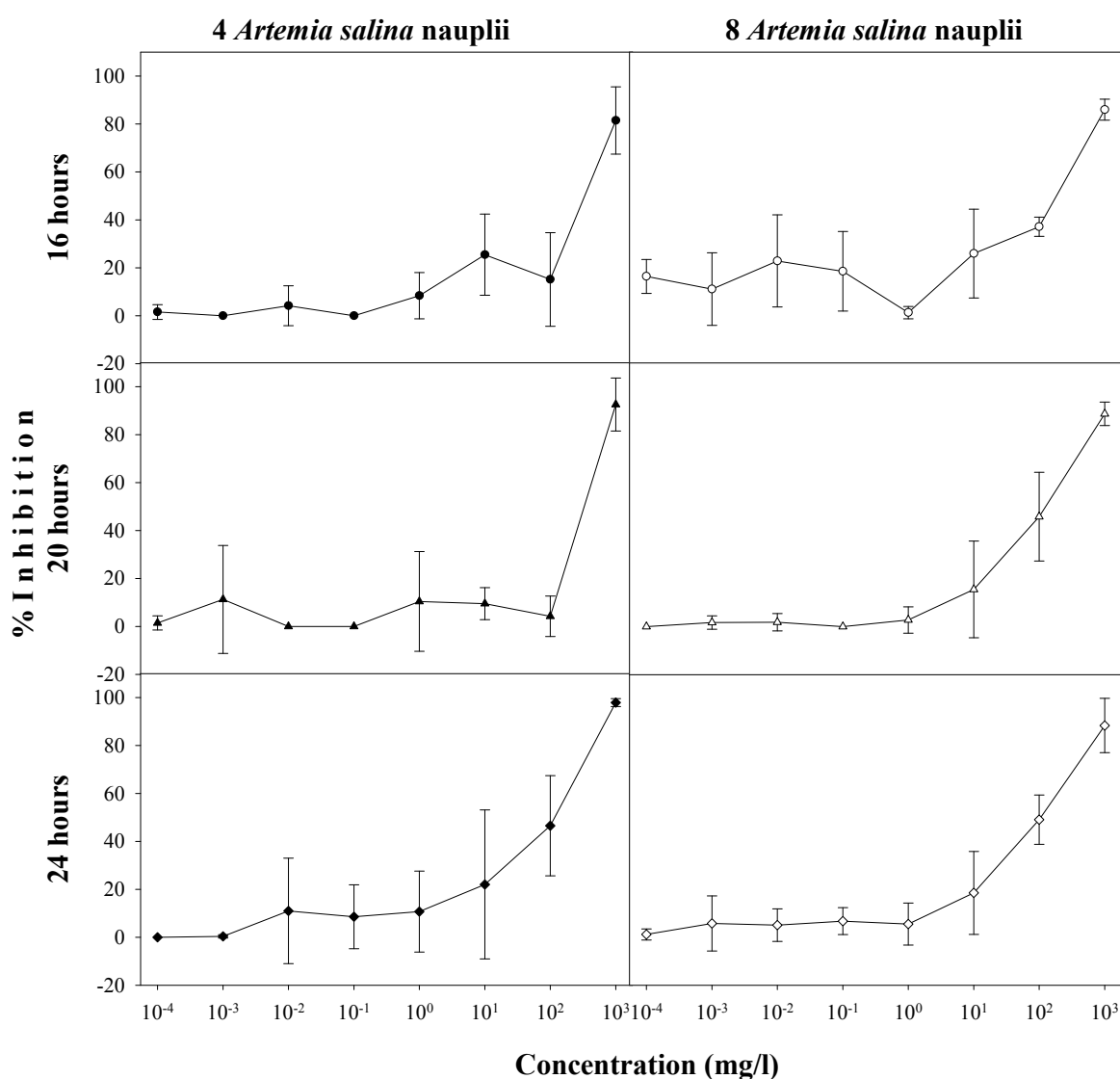
Figure 5.18 Percentage inhibition of the mobility of the nauplii after 16 (●) and (○), 20 (▲) and (△) and 24 hours (◆) and (◊) in zinc ion solutions with 4 nauplii (black symbols) and 8

nauplii (white symbols) respectively. The error bars represent the standard deviation of the mean of four replicates for each concentration.
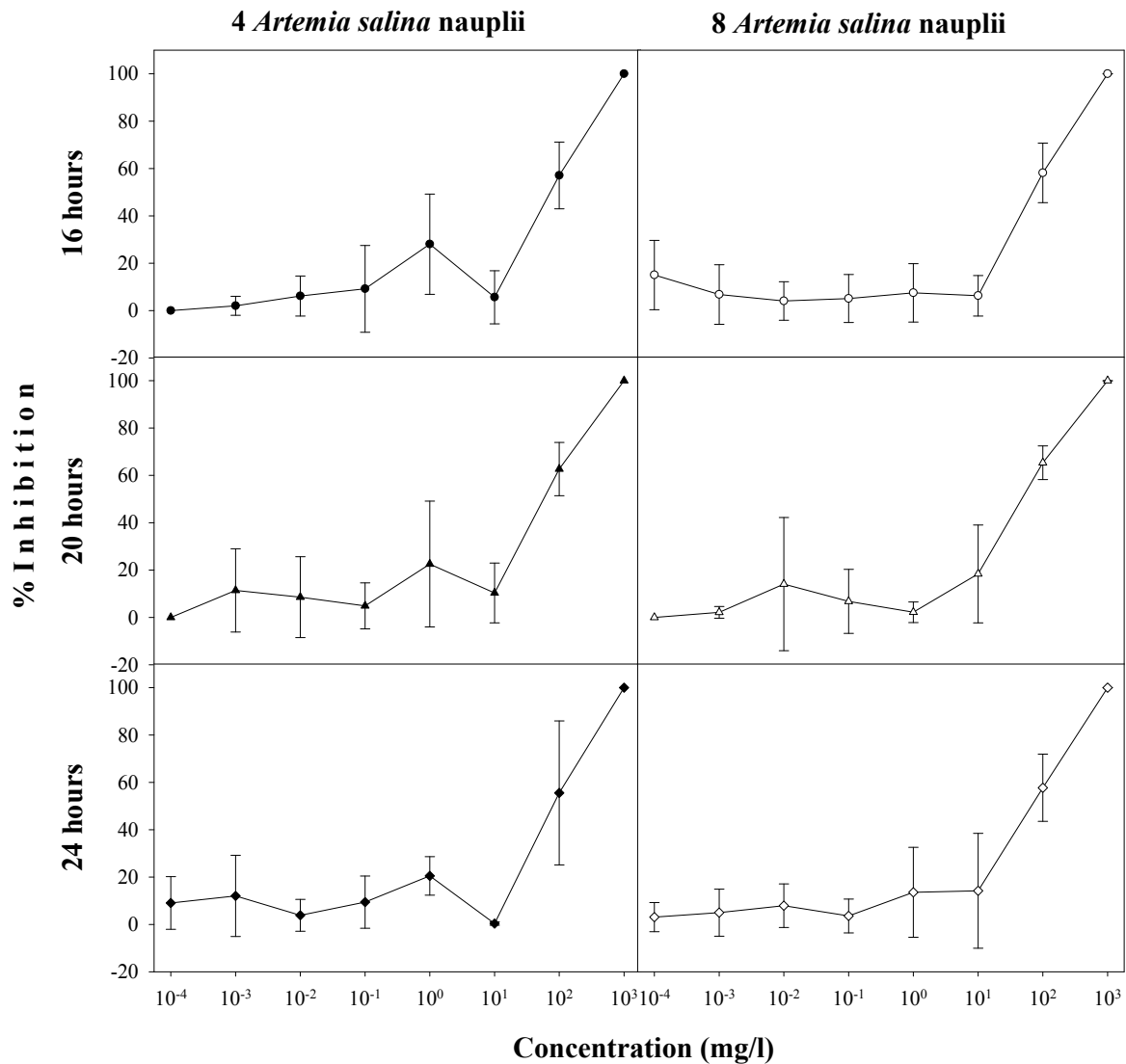
Figure 5.19 Percentage inhibition of the mobility of the nauplii after 16 (●) and (○), 20 (▲) and (△) and 24 hours (◆) and (◊) in phenol solutions with 4 nauplii (black symbols) and 8 nauplii (white symbols) respectively. The error bars represent the standard deviation of the mean of four replicates for each concentration.

The same analysis was also conducted for phenol solutions. The percentage inhibition of the mobility of the nauplii was significantly increased at the more concentrated samples according to Figure 5.19. More specifically, from the ANOVA analysis performed (Appendix A, Table A-55), the interaction among 'Concentration', 'Nauplii number' and 'Time' had P-

value higher than 0.05. So, the null hypothesis was accepted. This means that the mobility of both populations of nauplii (4 and 8) was inhibited in the same way when various concentrations of the toxic compound applied and when measurements were recorded in different time intervals.
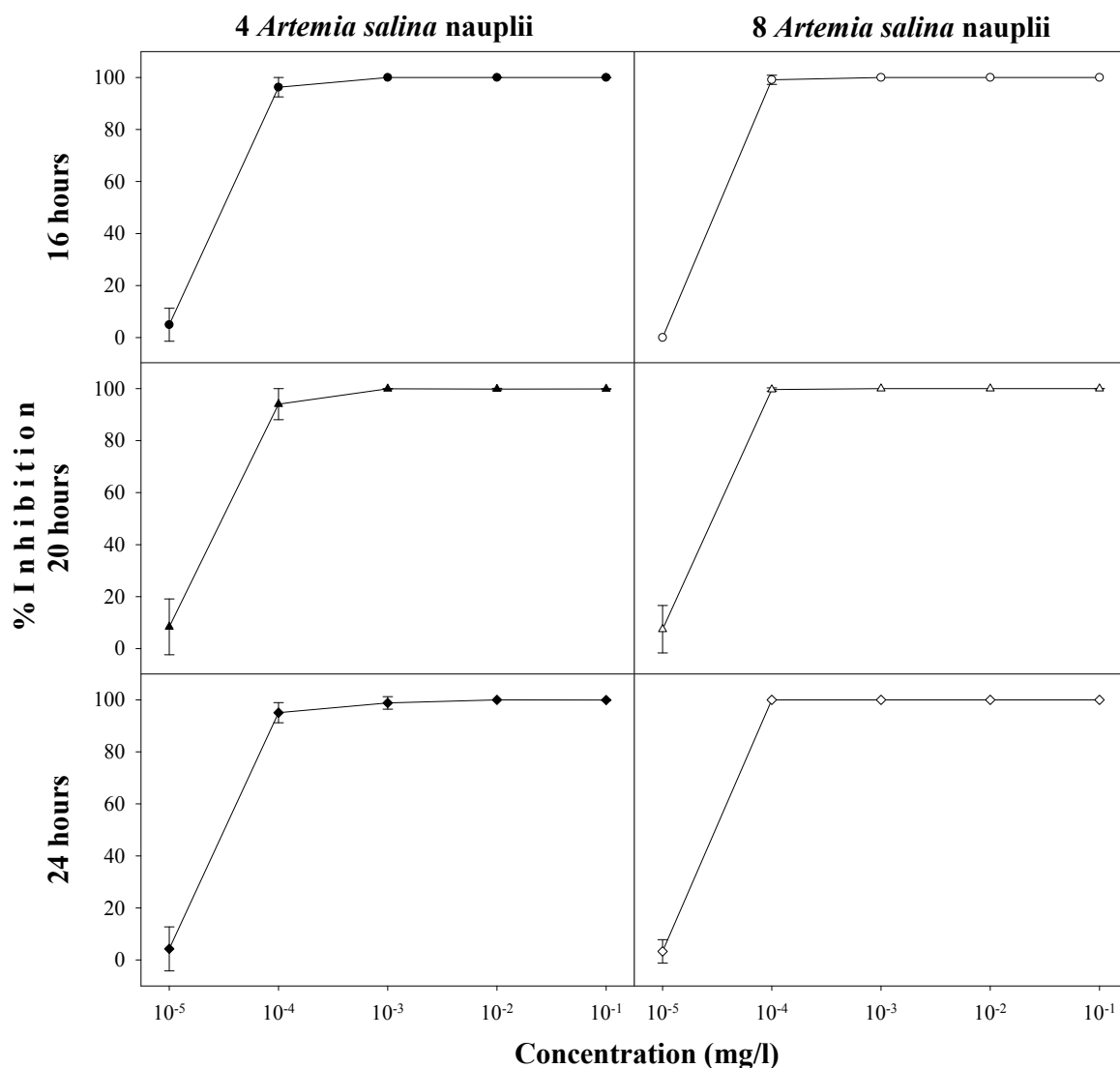
Figure 5.20 Percentage inhibition of the mobility of the nauplii after 16 (●) and (○), 20 (▲) and (△) and 24 hours (♦) and (◊) in sodium hypochlorite solutions with 4 nauplii (black symbols) and 8 nauplii (white symbols) respectively. The error bars represent the standard deviation of the mean of four replicates for each concentration.

Finally, the effect of sodium hypochlorite on the mobility of 4 and 8 *Artemia salina* nauplii at different exposure periods was analysed. The percentage inhibition of the mobility of the nauplii was significantly increased at the more concentrated samples according to Figure 5.20. Sodium hypochlorite did not affect the mobility of the nauplii at 10^{-5} mg/l but the rest of the tested concentrations 10^{-4}-10^{-1} mg/l inhibited approximately 100% of the mobility of the nauplii. More specifically, from the ANOVA analysis performed (Appendix A, Table A-56), the interaction among 'Concentration', 'Nauplii number' and 'Time' had P-value higher than 0.05 so the null hypothesis was accepted. Consequently, the mobility of both populations of nauplii (4 and 8) was inhibited on the same manner when various concentrations of the toxic compound applied and when measurements recorded in different exposure periods.

5.3.9 Development of a protocol using swarm of *Artemia salina* nauplii

The first methodology, as described in the section 5.2.9, was applied to cadmium ion solutions. A swarm of nauplii were inserted into the cuvette with the proper solution and videos were recorded at $time_0$ and $time_{24}$. The % inhibition was assessed and was significantly increased as the concentration became higher. The average inhibition of the mobility of the nauplii after 24 hours of incubation in various concentrations is illustrated in Figure 5.21. The rest of the tested concentrations inhibited the mobility of the nauplii in a higher degree than the controls.
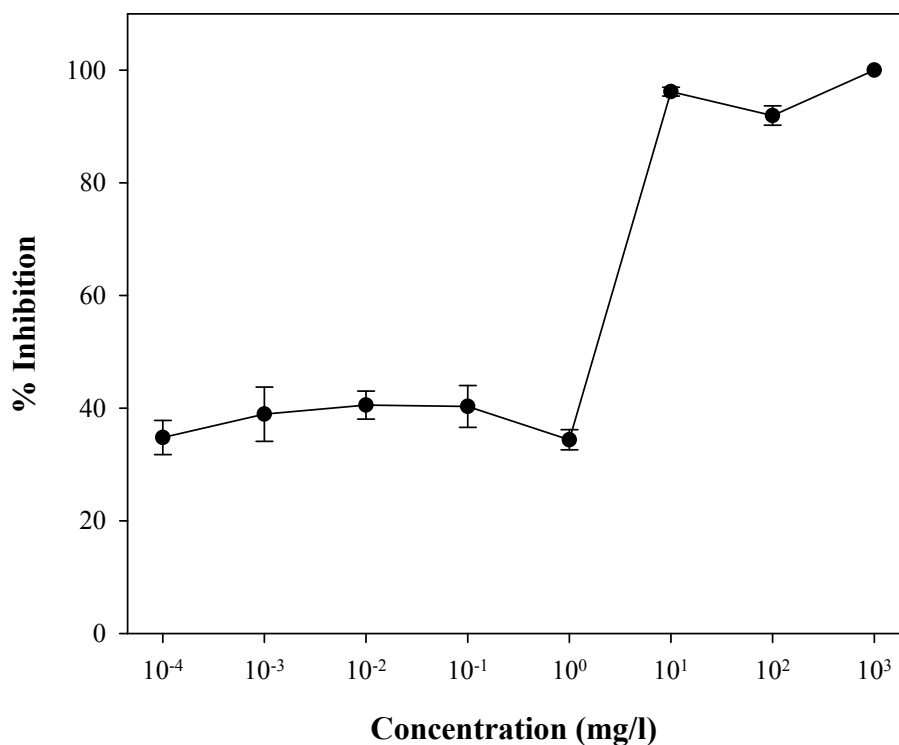
Figure 5.21 Percentage inhibition of the mobility of swarm of nauplii in copper ion solutions. The mean of four replicates and the error bars represent the standard deviation of the replicates with a $P < 0.001$ by analysis of variance.

In addition zinc and ferrous ions (Figures 5.22) were also tested with the same procedure as copper ions and the results were significantly increased at the most concentrated samples. Zinc ions did not influence the mobility at a high degree in comparison to ferrous ions where the most concentrated samples inhibited the mobility almost 100%.
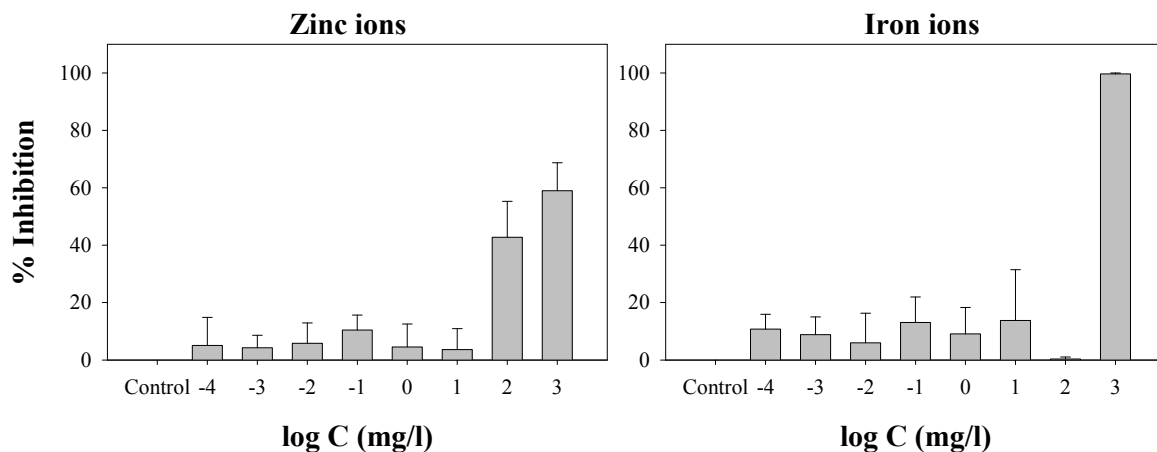
Figure 5.22 Percentage inhibition of the mobility of a swarm of nauplii in zinc and iron ion solutions comparing to the mean of controls. The values are the mean of four replicates and the error bars the standard deviation of the replicates with a P<0.001 by analysis of variance.

The second methodology applied initially to ferrous ions for 4 nauplii and a swarm of nauplii for the same tested concentrations (Colour plate 5.8). More specific range of concentrations was prepared based on the results of the previous sections. The results showed statistically significant increase of inhibition as the concentration increased (Figure 5.23). However, the swarm of nauplii showed low variance among the samples of the same dilution since the error bars are smaller than the ones of 4 nauplii. Moreover, from the curve of swarm of nauplii there is perspective of detection of toxicity to even lower concentrations.
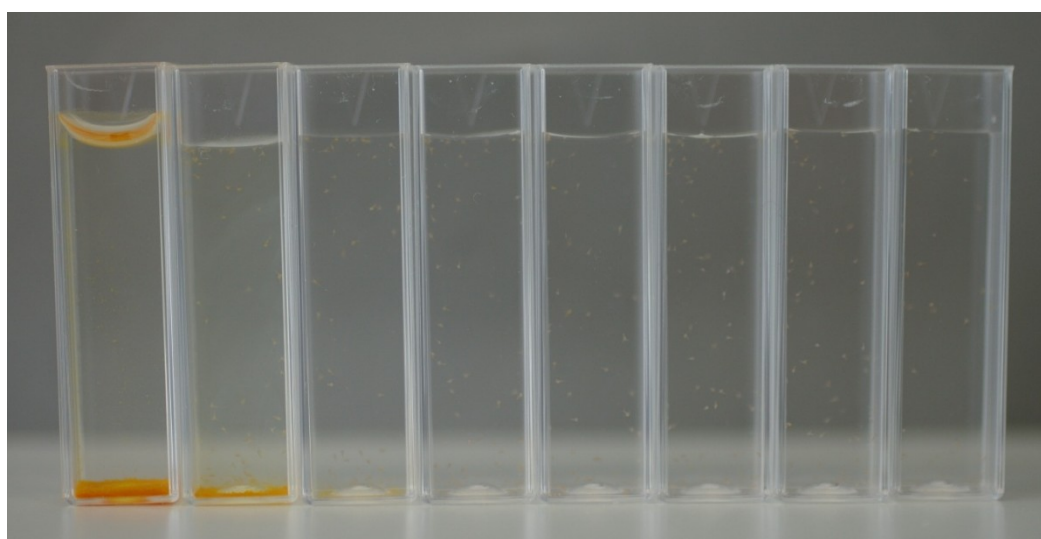
Colour plate 5.8 Dilutions performed for the toxicity assessment of Fe^{2+} with swarm of nauplii.
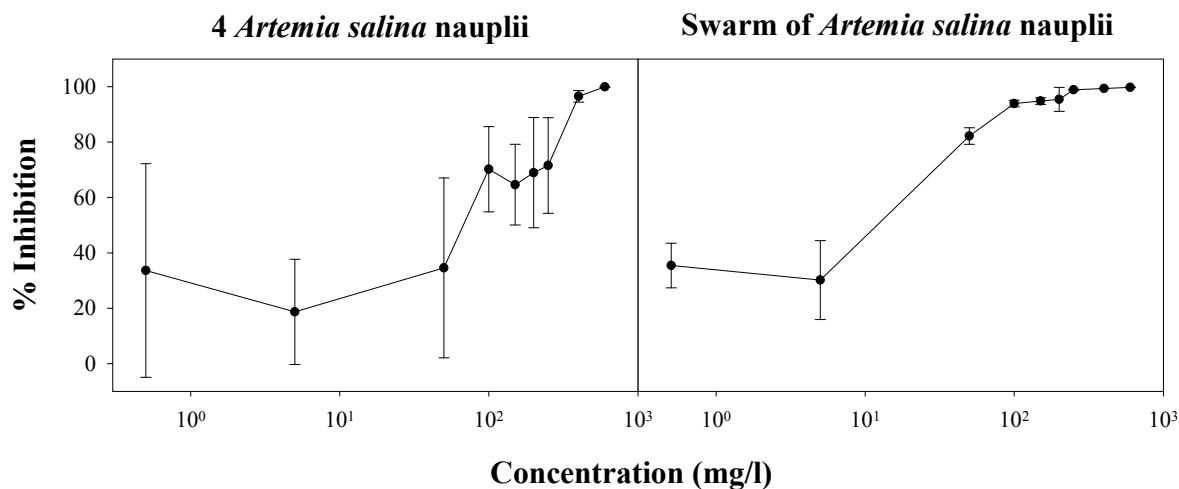
Figure 5.23 Percentage inhibition of the mobility of swarm of nauplii and 4 nauplii processed through the same protocol in iron ion solutions. The error bars represent the standard error of five replicates with a $P < 0.001$ by analysis of variance.

The second methodology was also applied to cadmium and copper ions to more specific concentrations as concluded from the previous experiments. The %inhibition significantly increased for both experiments as the concentration of the samples increased (Figures 5.24 and 5.25). Low levels of variance among the replicates were observed according to the error bars.

The IC_{50} values of all the tested compounds are illustrated in the Table 5.12 as estimated from the 'best fit model'.
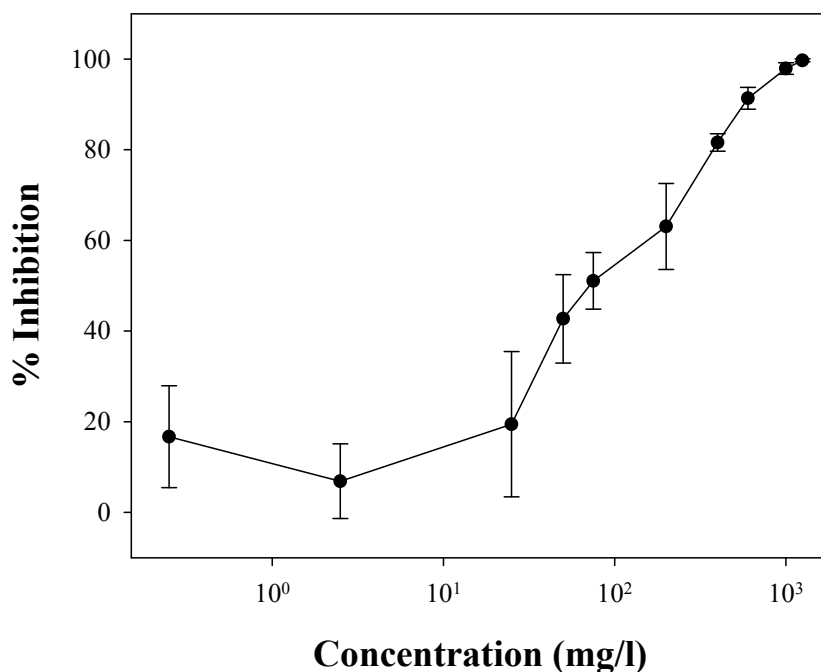
Figure 5.24 Percentage inhibition of the mobility of swarm of nauplii processed through the same protocol in cadmium ion solutions. The error bars represent the standard error of four replicates with a P < 0.001 by analysis of variance.
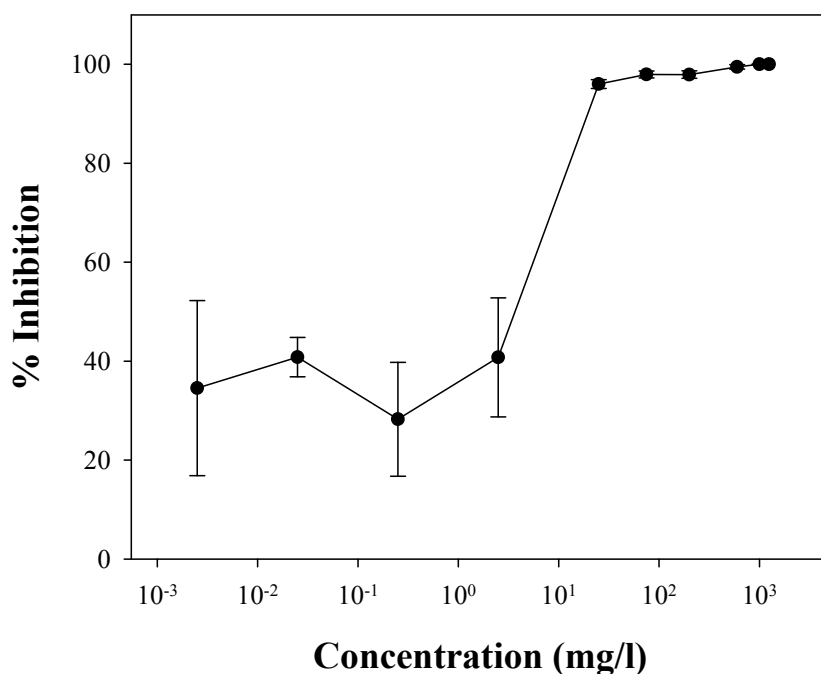
Figure 5.25 Percentage inhibition of the mobility of swarm of nauplii processed through the same protocol in copper ion solutions. The error bars represent the standard error of four replicates with a P < 0.001 by analysis of variance.

Table 5.12 IC_{50} values from the swarm tests and the protocol that was applied.

Compound (swarm)	IC_{50} (mg/l)	Equation from % inhibition graphs	Methodology
Copper	7.7	$y = -0.0005x^2 + 0.5611x + 45.695$ ($R^2 = 0.6262$)	1
Iron	821.6	$y = -0.0002x^2 + 0.1161x + 10.39$ ($R^2 = 0.993$)	1
Zinc	126.2	$y = -0.0004x^2 + 0.4072x + 4.9851$ ($R^2 = 0.9813$)	1
Iron 4 nauplii	91.6	$y = -0.0003x^2 + 0.2694x + 27.839$ ($R^2 = 0.9079$)	2
Iron swarm	11.5	$y = -0.0004x^2 + 0.3473x + 46.074$ ($R^2 = 0.7664$)	2
Cadmium	163.9	$y = -0.0001x^2 + 0.1853x + 22.308$ ($R^2 = 0.9203$)	2
Copper	NE* negative root	NE*	2

*NE: Not Estimated

5.3.10 Study of the effect of combination of metals on the mobility of *Artemia salina* nauplii

Since the toxicants are not found isolated in the environment, the effect of cadmium and copper ions on the mobility of *Artemia salina* nauplii was investigated. Initially, the effect of specific concentrations of cadmium and copper was separately tested in the previous section (5.3.9) and the results were utilised in order to analyse the combination effect to the mobility of the nauplii. According to Figure 5.26, the first group of tested compounds inhibited 100% the mobility of the nauplii when separately the toxic compounds provoke the same effects to the respective concentrations. In the second group, although the concentration of copper ions was very low, the toxic effect of cadmium ions predominated (independent effect). In the third group, where equal amounts of both compounds were added, the final effect was ranged approximately at the same levels as affected them separately, with slightly closer value to % inhibition from copper ions. In the following group, although the concentration of cadmium

ions was higher than coppers (600 mg/l and 25 mg/l), approximately the same high impact was provoked from both compounds separately and together. However, according to the results of the next group, the inhibitory effect caused by copper ions at 75 mg/l was higher than the effect caused by cadmium at 200 mg/l and was the same with the combinatory effect of both. So, again independent effect was observed. The next group was introduced to the nauplii with same concentrations but vice versa from the previous group. The same results were recorded with copper acting independently with no relation to the existence of cadmium. The three following groups, F, G and H, showed the same effects on the inhibition of the mobility of the nauplii with the previous ones. Copper ions toxicity was not influenced from the existence of cadmium ions. The same inhibitory effects observed as when copper ions acted alone. Finally, the last group with the lowest concentrations of both compounds, considering the error bars, the effect of copper ions was the same with the combination of both pollutants. So, again the effect was independent for copper.
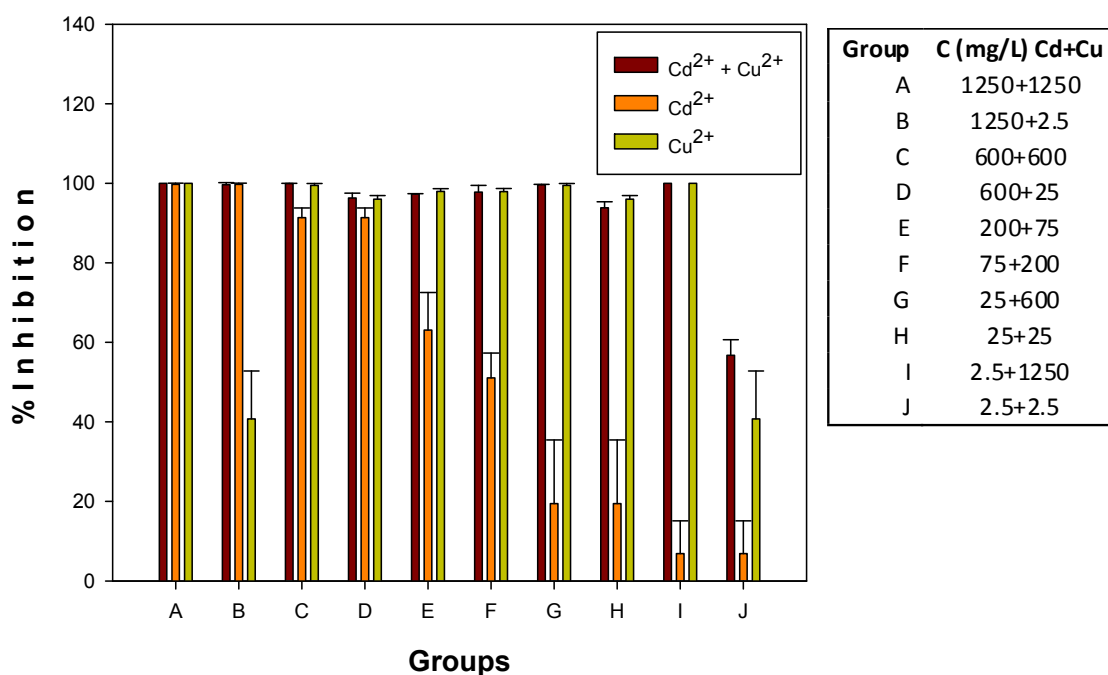
Figure 5.26 Percentage inhibition of the mobility of the nauplii in mixture of cadmium and copper ions at various combinations of concentrations and the relative effect of the specific concentrations to each contaminant. The error bars represent the standard deviation of three replicates.

5.4   Discussion

5.4.1   Mortality Based assay

The aquatic organisms are sensitive to abiotic characteristics of water such as salinity, pH, temperature, hardness and dissolved oxygen content (Song and Brown, 1998). Subsequently, the importance of retaining the proper levels of those characteristics was significant. So, the temperature was retained stable since the hatching temperature was 30 $^{\circ}$C inside the incubator and also the oxygen supply was constant. Regarding the salinity, the initial levels were 3.5 % but *Artemia salina* species is characterised as eyryhaline organism meaning that they are able to adapt themselves in high range of salinity levels. In addition, the pH values were evaluated before and after the incubation period of 24 hours with the toxic compound and they were always around the desirable levels ($\geq$7.5). Finally, the hardness was not further defined.

While all these parameters were verified, the experimental procedure was continued by counting the dead larvae inside the samples. The results are described in more details in the following section (5.4.2).

5.4.2   Study of the lethal effect of various toxicants on *Artemia salina* nauplii

The lethal effect of the reference substance of potassium dichromate and four other toxic compounds, atrazine, copper ions, phenol and sodium hypochlorite, were tested on *Artemia salina* nauplii. These compounds were chosen as representatives of four main categories of chemical compounds: herbicides, heavy metal ions, organic and inorganic compounds respectively.

Regarding the achieved results, atrazine did not show any particular effect on the vitality of the nauplii. This occurred due to low concentrations which were examined. Furthermore, atrazine is not a very water soluble substance, and this was a hinder to prepare more concentrated samples. The rest of the tested compounds showed high resistance levels since the LC_{50} values were greater comparing to the literature. Guerra (2001) estimated 24-hour LC_{50} of phenol at 17.3 mg/l using *Artemia salina* as test organism. Furthermore, Gajbhiye

and Hirota (1990) have studied the effect of heavy metals on *Artemia salina* nauplii and determined the 24-hour LC_{50} of copper at 9.5 mg/l. As far as the effect of sodium hypochlorite on the vitality of the nauplii, Sahul Hameed and Balasubramanian (2000) have reported that the 24-hour LC_{50} of sodium hypochlorite to instar-I *Artemia salina* nauplii was 5.6 mg/l. This value is quite low comparing to 207.8 mg/l obtained from this study which can be explained from the different age of the nauplii.

The high differences on the estimated LC_{50} values comparing to the literature might happen because of the application of ten-fold geometrically spaced concentrations. The choice of these concentrations is usually the first step for identification of the critical range of concentrations. Nevertheless, in the previous chapters these compounds at this specific range have been examined for their effect to plant cells and microorganisms. So, the continuation of the application of the same concentrations was necessary in order to compare the results from all the applied bioassays. Finally, the difficulty faced to count the dead nauplii in the test tube could also add to the possible error.

5.4.3 Mobility based assay

The results revealed that a novel screening system has been implemented with the capability of determining the toxicity levels when one substance exists in the solution. A step-by-step procedure followed and the factors affecting the performance of the device have been specified. Furthermore, this instrument was proved appropriate for defining the additive, synergistic, potentiation or antagonistic effect when two known substances subsist in aqueous solutions. In addition, the "best-fit" model was applied for describing the curve that represents the relation between the percentage inhibition and the concentration. After many trials, the best model with the minimum error proved the quadratic equation. However, this model did not well-describe the sharp curves such as the cases of colchicine and paraoxon-ethyl but only the smoother ones including the cases of aldicarb and thallium sulphate. As a result the R^2 was relatively low in some cases.

The results of the previous sections are further discussed, analysed and compared to the existing literature in the following sections.

5.4.4 Preliminary experiments with the initial device

These experiments aimed to show that the developed device had possibilities to be applied as a bioassay with relatively higher sensitivity than the mortality assay. In this section the results obtained were contrasted to those achieved from the section 5.3.2.

Although the percentage inhibition of the activity of *Artemia salina* nauplii in atrazine solutions did not significantly decrease, this device showed that can detect toxicity with higher sensitivity than is possible using the mortality assay. However, due to error and noise from the device, this advantage is not totally conclusive. The mobility monitoring of the nauplii showed that atrazine provoked deterioration of their movement ability compared to controls. This inhibition of mobility, though, was not very concentrated dependent. On the other side, the mortality assay showed that atrazine, at those specific concentrations, did not influence the nauplii. Consequently, the solutions were toxic according to the mobility assay but not toxic enough to kill the nauplii.

Moreover, the detection range of toxic effect of copper ions could be evaluated throughout the whole range of the examined concentrations. This could be translated by expansion of the detection scale to 100 times in comparison to the one from the mortality assay.

Regarding the phenol toxic effect, the range was again extended from 941 1mg/l, which was the first concentration where the nauplii started dying, to 9.4 mg/l which was the first concentration that provoked inhibition to the mobility of the nauplii. As a result, the range has been extended 1000 times between the two assays.

Finally, for sodium hypochlorite, the sensitivity range could be expanded more using this newly developed device. The percentage inhibition reached only the 41.6% at the lowest concentration (7.44 mg/l) which meant that even lower concentrations could be assessed in comparison to the mortality assay where at 74.4 mg/l all the nauplii died. So, the detection range of sodium hypochlorite can be extended more by applying the mobility assay.

To sum up, the firstly obtained results from this instrument showed great promise as a future bioassay. Higher sensitivity has been achieved through this device in detecting toxicity compared to the mortality assay (section 5.3.2). However, more improvements regarding both

the device and the process were essential in order to minimise the error and increase the accuracy.

5.4.5 Preliminary tests for optimising the final device

The aim of the experiments described in the respective previous section was to study the conditions that might affect the nauplii themselves during the assay and the performance of the device. The initial experiment performed in order to verify that the *Artemia salina* nauplii can retain their mobility for 24 hours at the same levels. The average mobility of five replicates with 8 nauplii was estimated at $time_0$ and $time_{24}$. The results showed that the average mobility remained constant. The same conclusion was reached every time that this experiment was performed as part of the verification process. This constitutes a promising result for continuing the experiments with this device since the main factor that can modify the mobility of the nauplii is the toxic compound.

Regarding the results from the reference substance, the LC_{50} value was evaluated using probit analysis and was found to be higher than the proposed range by Svensson *et al.* (2005). This happened due to the small number of tested organisms (four nauplii) inserted into the cuvettes for estimating the mortality effect.

A more specific experiment was performed in order to determine the optimum range of temperatures where the nauplii can be incubated for a maximum of 24 hours with their mobility remaining unaffected. This range proved to be between 15° C and 25° C. At both temperatures the mobility fell at the same level after 24 hours. The drop of the percentage mobility observed to all the examined temperatures was originated from the behaviour of the nauplii. During the recordings some of the nauplii were immobile on the walls or on the bottom of the cuvette. This behaviour firstly observed to this experiment and might happen due to regular interruptions for recording the samples (after 3, 6, 9 and 24 hours). Furthermore, the samples were recorded in open lab environment where the temperature is around 25°C. As a result, the change of the temperature in order the samples to be recorded, might also affect the activity and the general behaviour of the nauplii.

Regarding the experiments performed for the examination of the turbidity effect on the tracking sensitivity of the device, sand was applied. Sand is an element of the natural

environment of the nauplii which can alter the clarity of water without any toxic effect on their vitality. The gradual addition of sand in the samples increased the turbidity and absorbance. However, the % mobility of the nauplii was respectively decreased. Subsequently, the tracking ability of the algorithm deteriorated as the samples became more turbid. Controls of the same turbidity level but without nauplii were also recorded and their average value was subtracted from the samples (Equation 5.5) in order to minimise the error occurred from the particulate settling. However, the ability of the algorithm to track the nauplii in very turbid solutions reduced. Consequently, the clarity of the samples is a prerequisite for minimising the possible error.

In conclusion, the 24-hour incubation period did not affect the mobility of the nauplii. More specifically, the temperature was examined and it was testified that the nauplii retained their mobility for 24 hours between 15° C and 25° C. This comprises a very convenient result since no special adjustment of the temperature is necessary for conducting this assay. Furthermore, the reference substance, potassium dichromate, was effactually evaluated for its toxicity using the final device. Finally, it was proven that the tested samples must be of low turbidity in order for the nauplii to be successfully tracked.

5.4.6 Comparison of the toxicity of metal salts using the mortality and mobility based assays

The aim of the experiments described and performed (sections 5.2.6 and 5.3.6) was to compare in parallel the mortality and the mobility assay and evaluate their performance using various compounds. These experiments were conducted with metals since they are a common source of contamination in ecosystems. Additionally, iron was also considered due to its presence in the treated with Electrochemical Peroxidation (ECP) samples.

A high scale of concentrations was applied for all the substances for experiments, mortality and mobility assay, in order to specify the critical range of the sensitivity and especially regarding the newly developed device. As derived from the results, the critical range varied between the two experiments. The application of the mobility assay led to the detection of toxicity at lower concentrations that applying the mortality assay.

Furthermore, according to the results obtained with the mobility assay, the toxicity order of the tested compounds to *Artemia salina* nauplii was $Cu^{2+} > Cd^{2+} > Fe^{2+} > Zn^{2+}$. The established toxicity order to marine organisms regarding their most sensitive stages of life according to Waldichuk (1977) was $Cu^{2+} > Zn^{2+} > Ni^{2+} > Pb^{2+} > Cd^{2+} > Fe^{2+} > Mn^{2+}$, in which the toxic effect of Zn^{2+} differs from this study. However, Brown and Ahsanullah (1971) reported that the toxicity order in 1mg/l of the tested metal was $Cu^{2+} > Cd^{2+} > Fe^{2+} > Zn^{2+}$, which is the same as found here.

The effect of cadmium was estimated at low concentrations with this device. Organisms such as *Artemia salina* are used to accumulate cadmium ions and are considered very tolerant to cadmium exposure with LC_{50} values ranged from 93.3 mg/l to 280 mg/l (Sarabia *et al.,* 2002). Cadmium tolerance might be due to biological mechanisms include metal-binding and metallothionein synthesis (Sarabia *et al.,* 2006). However, applying the newly developed device the IC_{50} was estimated at the levels of 54.8 mg/l which is quite low compared to the LC_{50} range values.

Additionally, the LC_{50} values estimated with probit analysis were relatively high comparing to the literature. More specifically, the LC_{50} value for cadmium ions evaluated at 701.8 mg/l when Kissa et al. (1984) have estimated the 48-hour LC_{50} at 159.6 mg/l. Furthermore, the LC_{50} of copper and zinc ions to the nauplii were 19.5 mg/l and 1000 mg/l respectively while Gajbhiye and Hirota (1990) have determined the LC_{50} at 9.5 mg/l and 17.8 mg/l for copper and zinc ions respectively. This fact occurred due to the small population of the nauplii applied in each sample for assessing the lethal effect.

Moreover, during the experimentation process at the most concentrated samples of cadmium, iron and zinc ions precipitation was formed. However, the samples were not filtered in order to retain the initial concentration of the samples. The particulate settling did not affect the results since controls of every concentration was also prepared and recorded. The values of the controls were subtracted from the samples as has been described in the section 5.2.5 for reducing the error. Additionally, the most of the samples were colourful but this characteristic did not influence the tracking algorithm since the samples were not turbid.

In conclusion, after this comparative study the developed device proved more sensitive and with less error margins than the mortality assay.

5.4.7 Study of the toxic effect of pesticides and pharmaceuticals on *Artemia salina* nauplii

The main aim of these experiments was to examine the sensitivity of the device to various pesticides (aldicarb, thallium sulphate and paraoxon-ethyl) and pharmaceuticals (colchicine). These contaminants, apart from paraoxon-ethyl, are some of those required to be tested in order a device to be certified by US Environmental Protection Agency- Environmental Technology Verification (EPA-ETV) programme. More specifically, the determination of the threshold toxicity values of specific contaminants examined by a new device is a requirement in order novel technologies to be certified. The sensitivity of this newly developed device, implemented during this study, was determined by estimating the threshold toxicities to the aforementioned compounds and compared to the ones of Microtox$^{®}$, a verified technology. In addition, paraoxon-ethyl was also tested since it was the analysed compound of the most relevant patent by Portmann *et al.* (US Patent, 1998) regarding this device. Furthermore, during these experiments, two populations of *Artemia salina* nauplii were applied, 4 and 8, in order to find the most applicable population with the minimal error. The LC_{50} and IC_{50} values were also estimated.

Ten-fold, geometrically-spaced concentrations were applied for assessing the toxicity of these toxicants. Due to the implementation of a new device that no reference data exist, the need to identify its limits was imperative. Furthermore, according to literature when the mortality assay is applied, 10 ml of toxic compound and 10 nauplii are inserted in a Petri dish (Vanhaecke et al., 1981). By analogy, 4 nauplii were inserted in 4ml of toxic compound into the cuvette. The population of 8 nauplii was also applied for comparison reasons.

Regarding the aldicarb toxic effect, the critical range with either 4 or 8 nauplii for estimating the inhibition of their mobility was 1-1000 mg/l. The mortality effect could only be identified at 1000mg/l with 4 nauplii and between 100 and 1000 mg/l with 8 nauplii. Subsequently, the application of the final device revealed greater sensitivity than the mortality assay. Furthermore, the estimated IC_{50} values in this study were 34.8 and 61.9 mg/l with 4 and 8 nauplii (instar II-III stage) respectively after 24 hours of incubation.

The sensitivity range of the effect of colchicine to 4 nauplii was from 0.1 mg/l to 1000 mg/l when the inhibition of the mobility was monitored. This range was thousand times higher

compared to the evaluation of the lethal effect (100-1000 mg/l). In relevance to 8 nauplii experiment, the inhibition of nauplii movement was detected between 1 and 1000mg/l. Unfortunately, the results from the mortality assay were inconsistent with no specific reason. Consequently, the evaluation of toxicity was possible to less concentrated samples with 4 nauplii instead of 8. However, the IC_{50} values were close enough, 37.2 and 41.2 mg/l for 4 and 8 nauplii respectively.

Additionally, the impact of thallium sulphate to the movement of the nauplii was identified in a ten times lower range with 4 nauplii (1-1000 mg/l) than with 8 nauplii (10-1000 mg/l). A ten-fold shifted range was also defined between 4 and 8 nauplii when the lethal effect was estimated (10-1000 mg/l and 100-1000 mg/l respectively). Furthermore, the IC_{50} values were estimated to 34.1 with 4 nauplii and 42.4 mg/l with 8 nauplii.

Finally, the effect of paraoxon-ethyl was determined. The same critical range, from 1 to 1000mg/l using either 4 or 8 nauplii for both assays, mortality and mobility, was identified. Better results were recorded while estimating the LC_{50} values from the mortality effect. In addition, Portmann et al. (US Patent, 1998) has estimated the effect of paraoxon-ethyl to 12 *Artemia salina* nauplii when 12-36 images per sample were taken with resolution of 256 x 256 pixels. In the case of the device of this study, 30 frames per second were recorded for 2 minutes which ends to 3600 frames with resolution of 640 x 480 pixels. So, even though the EC_{50} value calculated by Portmann *et al.* (1998) was relatively low (0.27 mg/l), has not the degree of accuracy that the newly-implemented device offers.

The findings of this study regarding the LC_{50} values were relatively high for the most tested compounds. However, considering the LC_{50} and IC_{50} obtained values and the different endpoints that they represent, the LC_{50} values were expected to be higher. Although the critical range achieved with 4 nauplii was wider than with 8 nauplii, the variance among the samples according to the error bars was higher with 4 nauplii than with 8. Regarding the IC_{50} values achieved with 4 and 8 nauplii, they were mostly close enough to each other. However, the possible difference might be due to the higher sensitivity obtained when 4 nauplii inserted in the solution. A possible explanation is that the less the nauplii exist in the sample, the higher the quantity of the toxic compound per nauplius is consumed and consequently the higher the effect on their mobility.

Additionally, the threshold toxicities were also estimated in order to be compared to the ones achieved using the Microtox® and to human lethal doses. Microtox® is an approved

technology by US EPA-ETV programme. As displayed in the Table 5.13, the threshold toxicities values achieved with Microtox® technology ranged to the same level with the device developed during this study for aldicarb and thallium sulphate. Lower threshold toxicity value was achieved for colchicine using the newly-developed device. However, the threshold values were the same for both experiments with either 4 or 8 nauplii. Finally, lower values than the human lethal doses were estimated using the final device with *Artemia salina* nauplii apart from paraoxon-ethyl.

Table 5.13 Comparison of the threshold toxicity values (mg/l) of the tested compounds with the newly developed device using 4 and 8 nauplii, Microtox® and the human lethal doses (mg/l) respectively.

Compound	Threshold (mg/l) 4 nauplii	Threshold (mg/l) 8 nauplii	Microtox (mg/l)	Human Lethal dose concentration (mg/l)*
Aldicarb	10	10	28***	260
Colchicine	10	10	240***	240
Thallium sulphate	100	100	240***	2400
Paraoxon-ethyl	10	10	NA**	5mg/kg

* Concentration in 250ml of water, can be lethal to a person of 70kg if consumed

**NA: Not Applicable

*** Values adapted from http://www.epa.gov/nrmrl/std/etv/vt-ams.html#rtts

In conclusion, the aims of this section have been fulfilled since a new device has been developed with higher sensitivity than the commonly used mortality assay. Furthermore, the assay can be performed with either 4 or 8 nauplii with relatively less error with 8 nauplii but with higher sensitivity with 4 nauplii.

5.4.8 Study of the optimum exposure time of *Artemia salina* nauplii to the toxicants

The aim of these experiments was to minimise the exposure time of the nauplii from 24 hours to 16 hours. Subsequently, the impact of the toxic compounds on the mobility of *Artemia salina* nauplii was recorded after 16, 20 and 24 hours of incubation with the toxic compound. Furthermore, in order to testify if this reduction of exposure time is universal and can be applied to any toxic substance, six toxicants were applied; phenol, sodium hypochlorite and cadmium, copper, iron and zinc salts. In addition, two populations of nauplii were applied for each experiment in order to verify the results from the previous section (5.4.7).

In particular, the wide range of tested concentrations indicated statistically significant results for all the toxic compounds regardless of the number of the nauplii and the recording periods. However, there were no significantly different results among the tested variables of 'Concentration', 'Number of nauplii' and 'Time' for all the applied compounds. This means that even if 4 or 8 nauplii are used for each sample for the same range of concentrations the exposure time did not affected them.

To sum up, the toxic compounds had impaired the mobility of the nauplii even after 16 hours of exposure. Consequently, the bioassay's time can be reduced to 16 hours with the perspective of earlier detection. In addition, the tested populations were not influenced in different way by the contaminants compared to the results from the previous section (5.4.7).

5.4.9 Development of a protocol using swarm of *Artemia salina* nauplii

The main aim of the particular experiments was to develop a screening tool where the insertion of specific number of *Artemia salina* nauplii would be avoided and the assay could be run fast and easy. Subsequently, a swarm of nauplii was inserted in the cuvettes with either ASW or toxic solution. Ten-fold spaced concentrations were prepared during the first methodology of analysis of the samples but more specific concentrations were applied during the second one.

Videos were recorded at $time_0$ and $time_{24}$ for all the samples and the mobility at $time_{24}$ was divided by the mobility at $time_0$. This step performed for measuring the deterioration of the mobility of the nauplii existed in the sample. The same process was also applied with 4 nauplii in iron ion solutions but the results showed high variance among the samples. Furthermore, the determined IC_{50} value for 4 nauplii was approximately 8 times higher than the one achieved with the swarm of nauplii (Table 5.12).

In addition, the application of this device with a random number of *Artemia salina* nauplii revealed the possibility of detecting contaminants with high accuracy at very low concentrations. Specifically, in the case of copper, the device showed higher sensitivity meaning that the detection range of copper ions was extended to 10^{-4} mg/l. This range is very low since the maximum allowable concentration of copper is 0.112 mg/l for surface water and 1 mg/l for sewage water according to the UK government and the Water Quality Objectives (Dalley, 2001).

However, the aim of these experiments was to develop a quick and easy screening tool that could identify a potential hazard. The final methodology applied showed promising results. The application of this developed device to isolated toxic compounds proved effective and with high sensitivity in some cases. The next step was to apply the same method to mixtures of toxicants (section 5.4.10).

5.4.10 Study of the effect of combination of metals on the mobility of *Artemia salina* nauplii

The target of these experiments was to identify potential toxicity in mixtures of contaminants. The pollutants existent in the environment are found in many forms such as bound to other contaminants but rarely alone. The next step of the evolution of this newly developed device was to test it with mixtures of compounds. The mixture applied was Cd^{2+} with Cu^{2+} due to their relevant high toxicity in many aquatic organisms. In general, Cu^{2+} has been reported as more toxic than Cd^{2+} to *Cyprinus carpio* (carp) and to *Oncorhynchus mykiss* (rainbow trout), which are both freshwater fish species (Ługowska, 2007). Furthermore, the same findings were also reported when these two metals applied to cyprinid fish embryos (Kapur and Yadav, 1982).

In this study the findings showed that in the most combinations tested Cu^{2+} acted independently. However, when the dilutions were very high for both contaminants the effect remained at the same levels as it was for each one separately. Furthermore, when the concentration of Cd^{2+} was much higher than Cu^{2+}, Cd^{2+} behaved independently in that mixture. The most of the rest combinations applied, the results showed the independent action of Cu^{2+} even to the least concentrated samples. In general, Cu^{2+} showed higher impact comparing to Cd^{2+} on the mobility of the nauplii.

5.5 Conclusions

The application of two endpoints of *Artemia salina*, mortality and mobility, in order to assess the toxicity levels of aqueous solutions was performed. During the mortality assay, representatives of four main categories of toxic compounds, Atrazine-herbicide, copper salts-heavy metal, phenol-organic compound and sodium hypochlorite- inorganic compound, were tested. The results showed the expected pattern to all the applied compounds with the exception of Atrazine.

Then, a device was implemented for monitoring the mobility of *Artemia salina* nauplii as an indication of toxic effects of various harmful compounds. Videos were recorded and analysed using digital image processing for tracking the nauplii inside the toxic solution with high accuracy. The results, confirmed the hypothesis of this chapter which was that more sensitive results can be obtained using this device than the mortality assay. Various contaminants were applied and many experiments performed in order to establish the working protocol using this device. Initially, it was justified that the nauplii could retain their activity for 24 hours in their natural environment. Afterwards, the working temperature for incubation and recording the videos was confirmed that it could be the lab temperature (25°C) and the turbidity levels must be very low for more accurate results. Furthermore, the most appropriate population between 4 and 8 nauplii and the minimisation of incubation period attempted to be established. However, the results revealed that there was no significantly difference either between the ways that both populations acted or the exposure periods among 16, 20 and 24 hours. However, swarm of nauplii was also indicated in order to simplify the assay and develop a quick assay. The results revealed that the higher the population the lower the error. Though,

more experiments are necessary in order to verify if the sensitivity levels and consequently the detection limits are changing when more nauplii exist in the solution. Finally, a combination of metals, cadmium and copper, were successfully applied for evaluating their effect on the nauplii.

In conclusion, a novel device has been developed which tracks successfully the mobility of the nauplii at lower concentrations than the mortality assay as proved from the aforementioned experiments. Additionally, it is proved applicable to a wide variety of contaminants with results often better when compared to other competitive technologies.

Chapter 6 Electrochemical peroxidation (ECP) process and toxicity assays

6.1   Introduction

Various wastewater remediation treatments have been established in order to improve the protection of the environment from pollution. More specifically, the ECP process has been applied over the last two decades for treating wastewater. However, issues regarding the toxicity of wastes prior to, and following the treatment, have been raised. The increasing demand for preventing environmental disasters such as Buncefield fire in UK and mine spill in Spain has led to stricter European regulations regarding the maximum limits of various contaminants.

In particular, twenty years ago the quality of the effluents was based on parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD) and total suspended solid (TSS) (Directive 91/271/EEC, 1991). Later, the guidelines of the Directive 2000/60/EC and Decision 2455/2001/EEC provided a list of priority of organic contaminants including antifouling agents and pesticides. In addition, more recently, the number of reports concerning pharmaceutical compounds in surface water has been increased and as well as the demand for monitoring the water quality (Hernando *et al.,* 2005).

Regarding landfill leachate and industrial wastewater, the assessment of a potential hazard is usually performed with either chemical analysis or toxicity assays based on one organism or a battery of toxicity assays sensitive to different groups of contaminants. During the previous decades, chemical analysis has proven poorly suited to the evaluation of the contaminants present in wastewater and their toxic effect on the environment (Bernard *et al.,* 1996). As a result, the application of bioassays started being applied but with only one type of species such as fish, microalgae or crustaceans for estimating the toxicity of landfill leachates (Walker and Andrian, 1977; Vigers and Ellis, 1977; Cameron and Koch, 1980; Cheung *et al.,* 1993). Furthermore, Srivastava *et al.* (2005) estimated the toxicity of leachates using onion bulbs of *Allium cepa* and measuring the effect on their development and, hence, their weight.

Svensson and co-researchers (2005) investigated the effect of leachates from three different landfills on *Artemia salina* nauplii (instar stage II-III).

In the meantime, some researchers applied a limited set of different types of organisms for assessing the toxicity of landfill leachate including fish, microorganisms and microalgae (Atwater *et al.,* 1983; Ernst, 1994; Jean and Fruget, 1994). Moreover, Bortolotto *et al.* (2009) determined the toxicity and genotoxicity of landfill leachates in southern Brazil applying as test species *D.magna*, *Artemia* species, *A.cepa* and *Geophagus brasiliensis*. Other researchers combined the chemical analysis of effluents to a toxicity bioassay in order to assess the toxicity level of groups of contaminants. In particular, Reemtsma *et al.* (1999) analysed various industrial effluents and assessed the toxicity throughout the analytical procedure using *V.fischeri*. Furthermore, Isidori *et al.* (2003) evaluated the toxicity of two municipal solid waste landfills in southern Italy by using the U.S. Environmental Protection Agency (EPA) developed toxicity identification evaluation protocol in order to detect and identify the existent toxic compounds. Subsequently, the toxicity of the contaminants was determined by applying organisms living in the freshwater including the rotifer *Brachionus calyciflorus*, the crustaceans *Thamnocephalus platyurus* and *Daphnia magna* and the bacterium *V.fischeri*.

Over the past decade, or so, the application of a great variety of toxicity assays to wastewater has increased. The majority of these studies dealt with untreated wastewater. However, the demand for reducing the toxicity of samples has increased and many researchers started applying remediation treatments and evaluating the process using toxicity assays prior to and after the treatment. For example, Palácio *et al.* (2009) treated textile dye wastewater using an electro-coagulation process and assessed the toxicity of the untreated and treated effluent utilising *Artemia salina* and lettuce seeds (*Lactuca sativa*). Furthermore, Farré *et al.* (2001) determined the toxicity of treated industrial wastewater and untreated effluent from textile industry form Spain and Portugal using ToxAlert[®] 100 and Microtox[®].

To sum up, landfill leachates are complex effluents with various contaminants which ideally need a battery of assays of different organisms with specificity to different toxicants for assessing their toxicity. In addition, the application of a battery of assays increases the reliability of the result achieved only with one bioassay. A proper combination of toxicity assays must be performed in order to fulfil some criteria including the choice of representative organism, the sensitivity of the assay and the cost. Little attention has been paid to these criteria and subsequently the number of the studies which combine a wastewater

treatment and the evaluation of toxicity of the untreated samples, their toxicity during the treatment and after the process are low enough.

Subsequently, the work presented in this chapter addresses the combination of an advanced oxidation process, the ECP, with toxicity assays applied to the samples prior to the treatment, during the process and at the end of the process. The chloroplast assay (Chapter 3), the microbial assay (Chapter 4) and the invertebrate assay (Chapter 5) were applied to landfill leachate samples which were collected from Donarbon Ltd (Waterbridge, Cambridgeshire) and from Cranfield's wastewater treatment plant.

6.2   Materials and methods

6.2.1 ECP with chloroplast assay

The materials and methods that were applied for the following experiments were extensively described in section 2.2.1 for the ECP process and in sections 3.2.1-3.2.4 for the chloroplast assay. The experimental parameters on the samples before chloroplast assay being applied are discussed in the following sessions in detail.

Storage time of compost leachate samples on toxicity

Compost leachate samples from run B were tested using the chloroplast assay. This was performed on part of the samples straight after ECP process and repeated after 3 days of storage (Figure 2.3). During this period of time, they were stored in a fridge at 4°C. The effect of low temperature storage on the chloroplast assay was assessed.

Sterilisation of compost leachate samples on toxicity

Two samples were taken for each period of time before and during the ECP process from the run C (Figure 6.1). These were split into two batches and one group of those was sterilised by autoclaving at 121°C for 15 minutes. Both parts were tested for their toxicity with the chloroplast assay using sequential dilutions.

Sterilisation was used as a further pre-treatment in order to avoid the presence of microorganisms, which may affect the photosynthesis and thus, inhibit the chloroplast assay. Several dilutions were applied but the most appropriate proved the 10^{-2}.
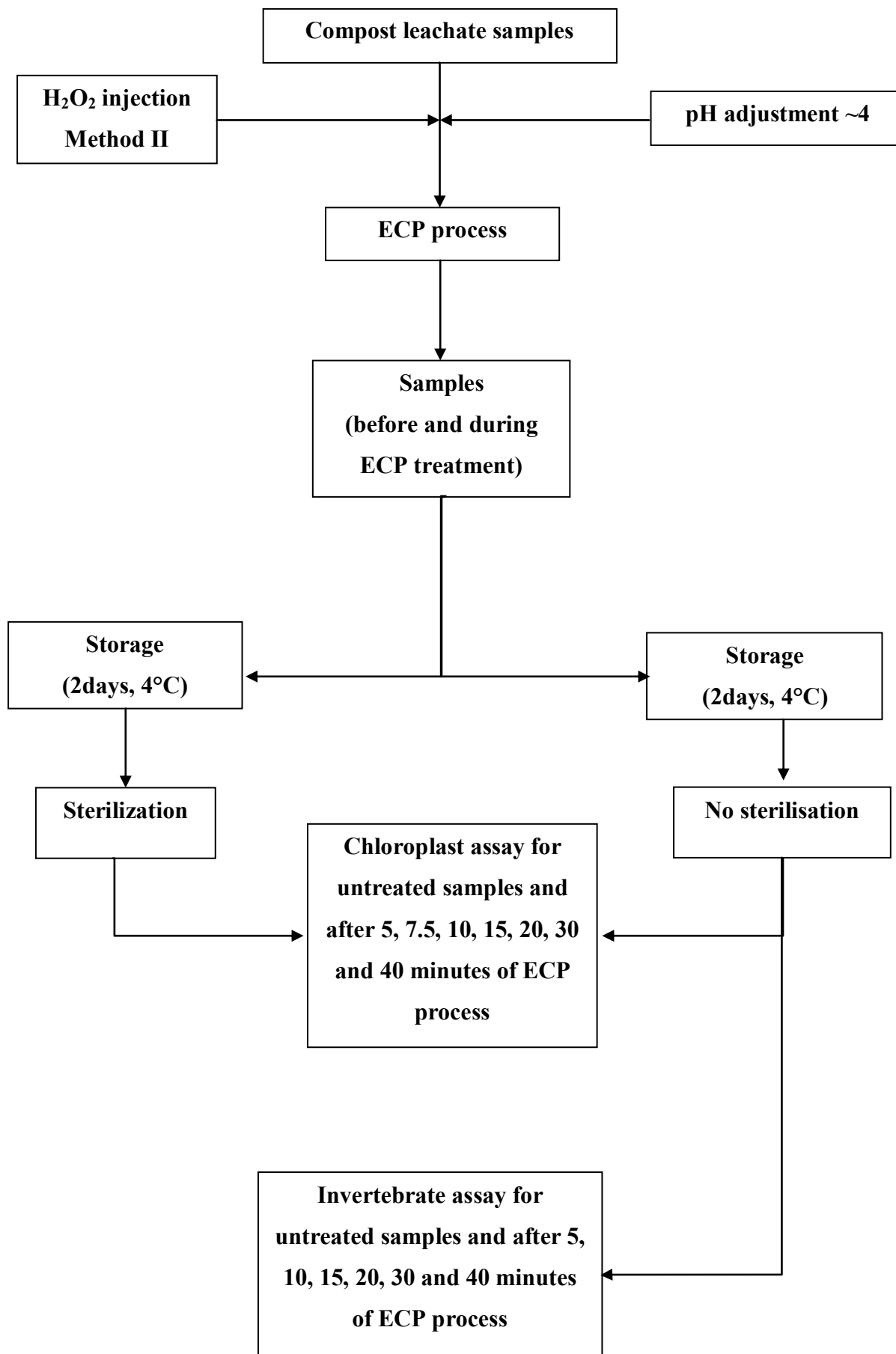
Figure 6.1 Flow diagram of run C experimental procedure.

6.2.2 ECP with microbial bioassays

Two ECP Runs were performed for estimating the toxicity using the growth inhibition of *E. coli, P. aeruginosa* and *S. aureus*. Leachate samples from the sewage treatment plant in Cranfield (UK) (run D) and compost leachate samples from Donarbon (Waterbridge, Cambridgeshire) (run E) were treated using ECP (Figure 6.2). Samples were obtained prior to the treatment and at 5, 15 and 40th minute of the treatment in 10 ml sterilised universal bottles. Then, the disc susceptibility assay was applied as described in section 4.2.1 for estimating the growth inhibition of *E. coli, P. aeruginosa* and *S. aureus* for the run D (in duplicate) and the growth inhibition of *E.coli* for the run E. However, instead of saturating the disks with toxic compounds, they were dipped in wastewater samples. The methodology was described in detail in section 4.2.1.

6.2.3 ECP with invertebrates

Compost leachate samples provided by Donarbon (Waterbridge, Cambridgeshire) were used for determining the toxicity of wastewater samples using *Artemia salina* nauplii (instar stage II-III) after being treated using ECP. An extensive sampling procedure was performed during run C where samples were obtained prior to the treatment and after 5, 10, 15, 20, 30 and 40 minutes of ECP process. The toxicity of these samples was evaluated with 8 nauplii per sample. During the next two runs (E and F), the toxicity of untreated and after 40 minutes of ECP process treated samples were evaluated using 8 *Artemia salina* nauplii per sample. The samples were diluted 60% using artificial seawater (ASW) 3.5 ± 1 %. The pH was adjusted to values higher than 7 and the appropriate quantity of artificial sea salt was added in order to reach the appropriate level of salinity for the nauplii to survive. The hatching of the cysts was analytically described in the section 5.2.1 and the performed methodology was described in the section 5.2.3.
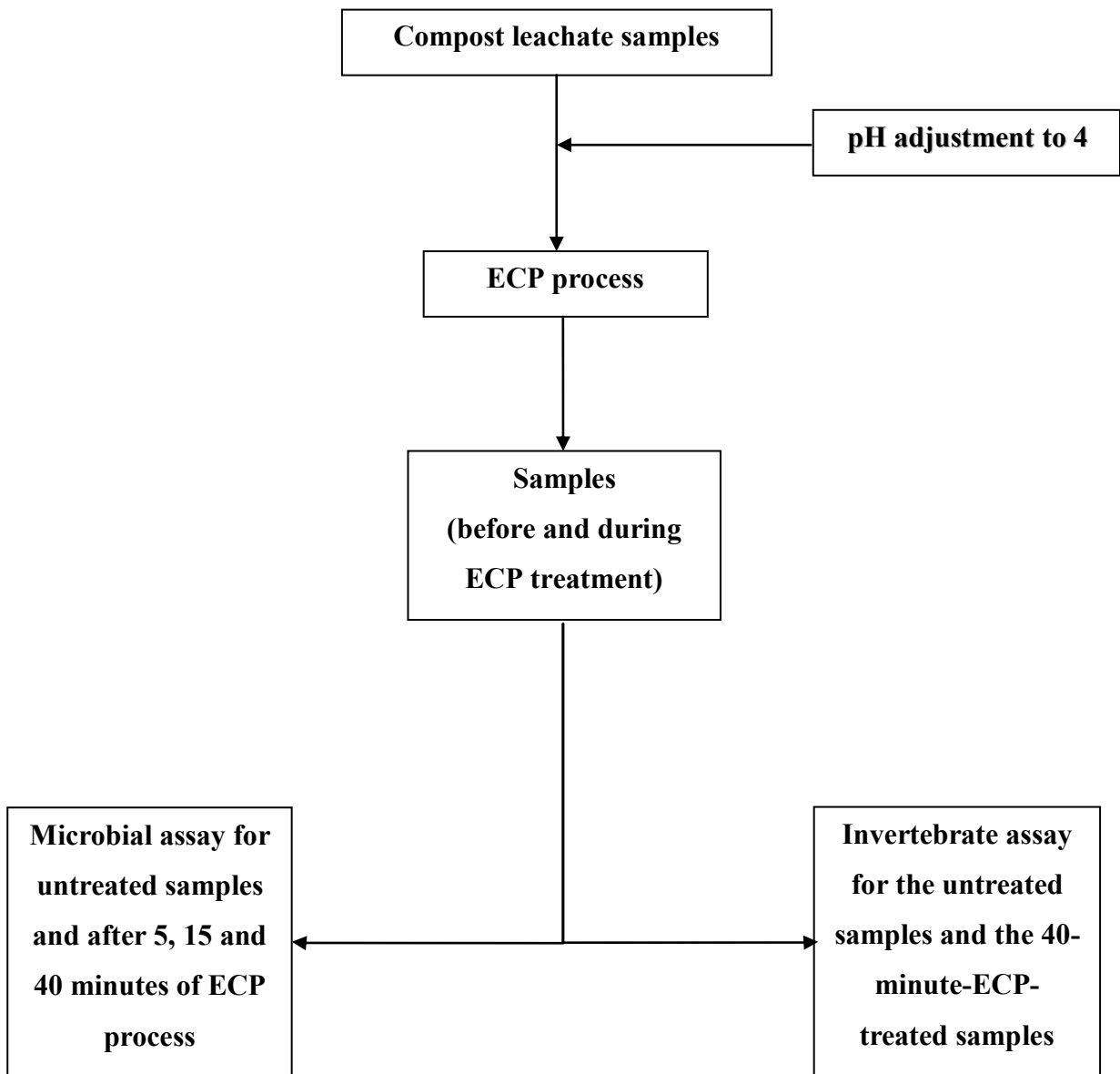
Figure 6.2 Flow diagram of run E experimental procedure.

Table 6.1 Experimental overview.

	Run B	Run C	Run D	Run E	Run F
Chloroplast assay	✔	✔	✘	✘	✘
Microbial assay with (*E. coli,* *P. aeruginosa* *S. aureus*)	✘	✘	✔	✔	✘
Mortality of *Artemia salina* nauplii	✘	✔	✘	✔	✔
Mobility of *Artemia salina* nauplii	✘	✔	✘	✔	✔

6.3 Results

6.3.1 ECP with chloroplast assay

Storage time of the compost leachate samples on toxicity

Samples from the experimental run B were tested for their toxicity straight after the ECP process and after three days of storage in the fridge (4°C). The samples (pH 4) were filtered before the application of the toxicity assay. The most representative dilution proved to be 10^{-2}. The following graph (Figure 6.3) represents the results without storage and after 3 days storage.
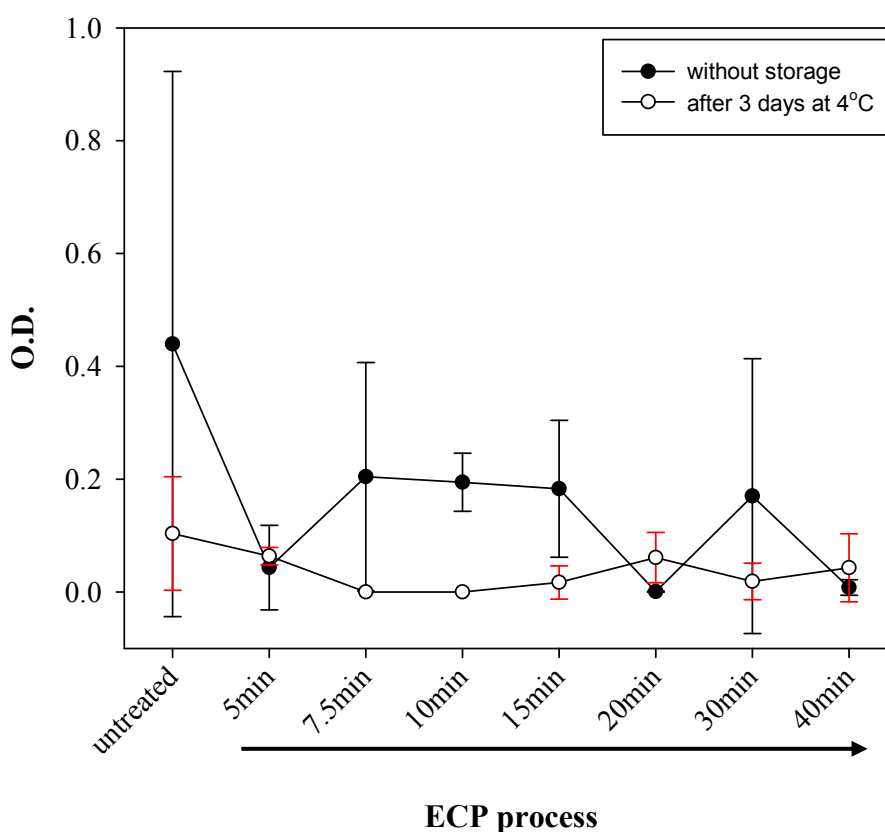
Figure 6.3 Effect of storage time of 3 days (fridge, 4°C) on toxicity of the same samples (compost leachate samples) from the run C at the dilution of 10^{-2} prior and during the ECP process using the chloroplast assay (550nm). The error bars represent the standard deviation of three replicates.

An inconsistent level of toxicity was observed for both parts of the samples. However, the absorbance values and consequently the toxicity were generally lower for the samples stored for 3 days in the fridge. Furthermore, the toxicity levels at the end of the process, for both batches, reached almost the zero level. In addition, since the effect of atrazine to chloroplast assay reached the O.D. values of 1.2 and considered toxic, these wastewater samples untreated or treated, stored or not, were not considered toxic. Consequently, the specific compost leachate samples were not toxic to chloroplasts either prior to the treatment or at the end of it.

Sterilisation of compost leachate samples on toxicity

The effect of sterilization was studied on the samples collected from experimental run C. Figure 6.4 displays the plots of the O.D. values (550nm) achieved from the chloroplast assay from the sterilised and non-sterilised samples at the dilution of 10^{-2}. The O.D. values and subsequently the toxicity levels followed an inconsistent pattern during the ECP process. This fact can be explained by the degradation of the existent recalcitrant compounds existent in wastewater samples which occurred during the treatment. Finally, the toxicity varied at the same low levels for both parts of sterilised and non-sterilised samples.
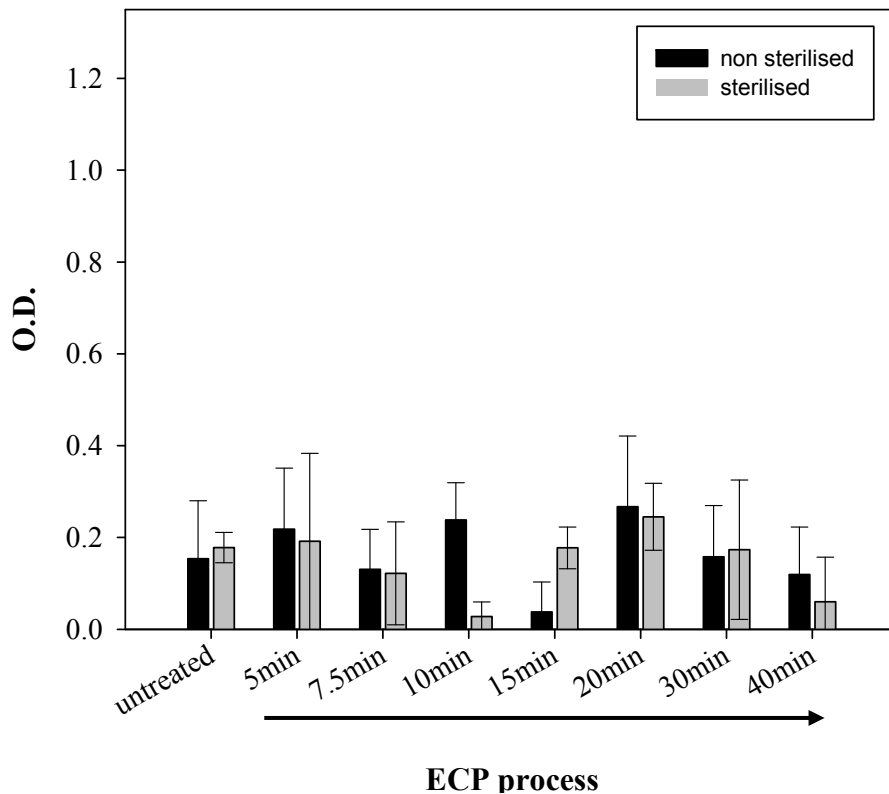
Figure 6.4 Chloroplast assay for the non- and sterilised leachate samples of the untreated samples from the run C at the dilution of 6 x 10^{-1} prior and after 5, 7.5, 10, 15, 20, 30, and 40 minutes of ECP treatment (550 nm). The error bars represent the standard deviation of three replicates.

6.3.2 ECP with microbial assay

The results from the disc susceptibility assay from the run D showed that the ECP treatment did not increase/change the toxicity of the wastewater samples. In particular, the growth of the cultivated microorganisms was not inhibited from the wastewater samples treated or not.

The same results were obtained from the application of the microbial assay of *E.coli* to compost leachate samples (run E). The samples were obtained at the same time intervals with the previous run (D) and the growth of *E.coli* was not inhibited by the existence of either untreated samples (Colour plate 6.1) or during treatment or at the end of the treatment (Colour plate 6.2).
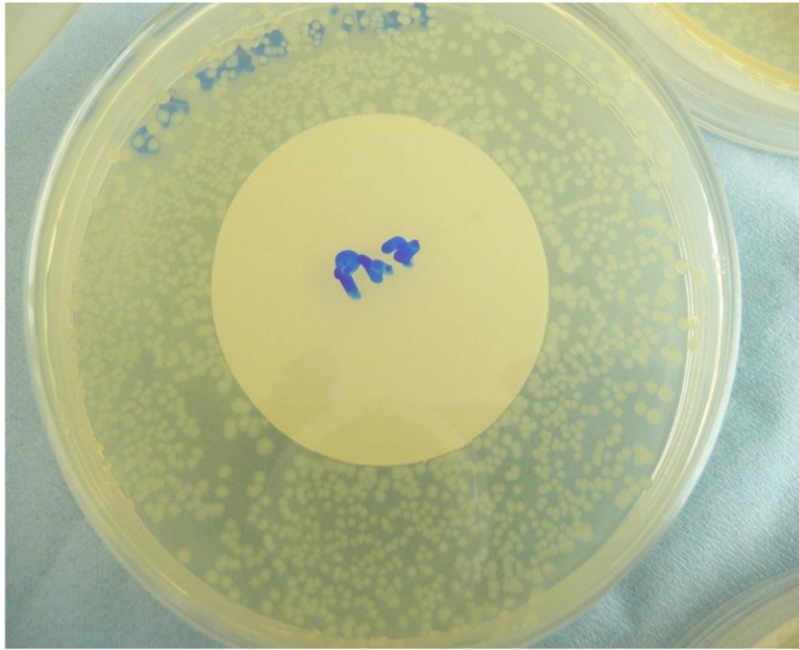
Colour plate 6.1 Saturated disc with untreated wastewater sample of pH 7 on bacterial cultivation (Murphy, 2009)
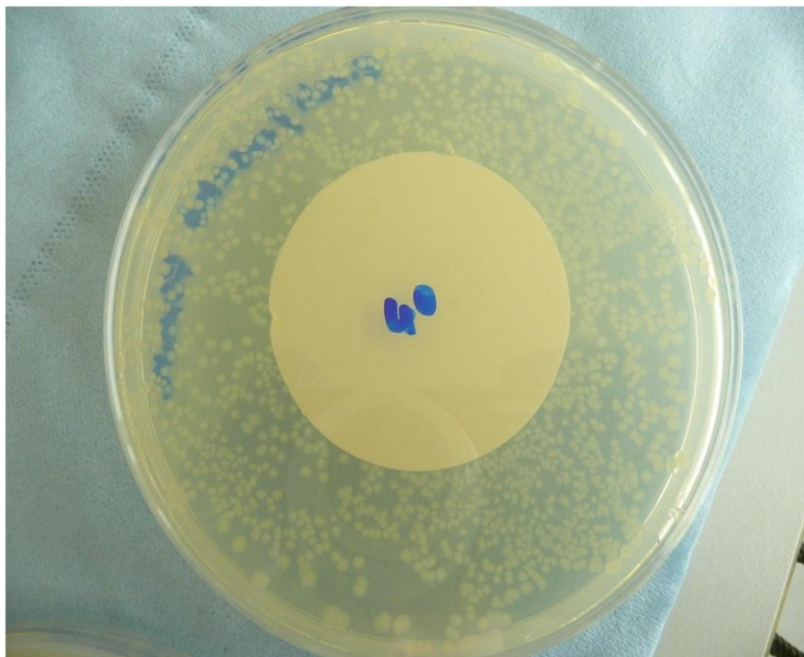
Colour plate 6.2 Saturated disc with 40-minute-ECP-treated sample of pH 7 on bacterial cultivation (Murphy, 2009)

6.3.3 ECP with invertebrates

The toxicity of the samples of the run C was not only evaluated with the chloroplast assay but also with the mortality and mobility assay of *Artemia salina* nauplii (instar stage II-III). Initially, the proper amount of sea salt was added to the samples and 4 ml of the solution were inserted into the cuvette diluted by 60% with ASW. The results from the mortality assay showed that all the nauplii survived after the incubation period of 24 hours in the wastewater samples and the ECP-treated ones. However, the results obtained from the mobility assay showed that the toxicity of the samples followed an inconsistent pattern. However, at the end of the process the toxicity of the samples decreased since the percentage inhibition of the mobility of the nauplii reached the level of the control (Figure 6.5).
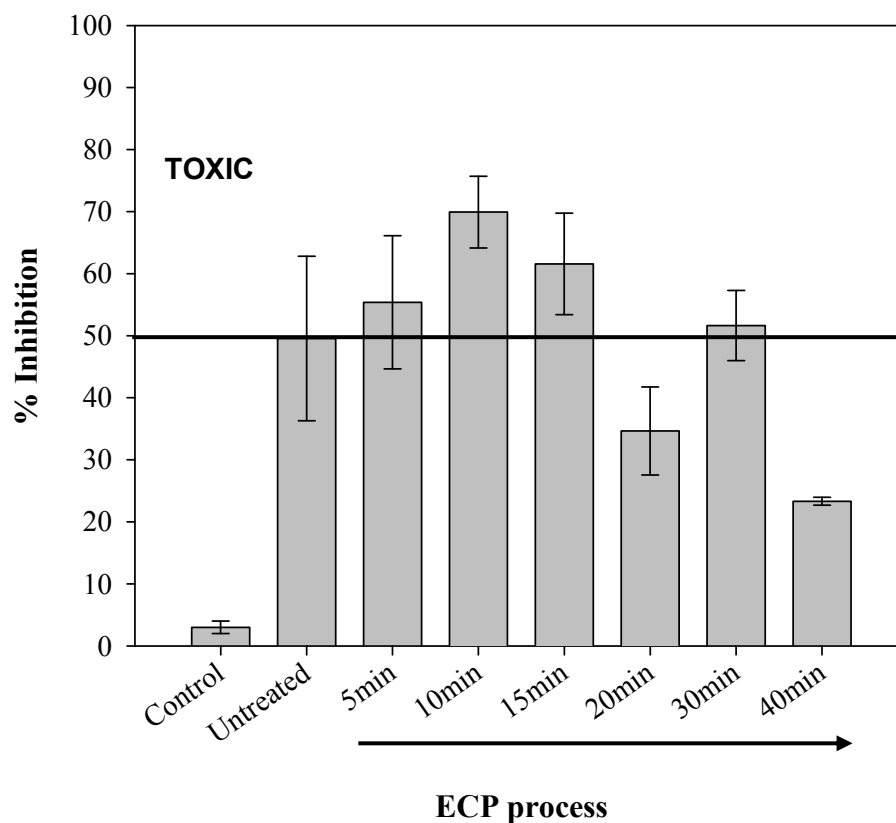
Figure 6.5 Percentage inhibition of the mobility of *Artemia salina* nauplii in compost leachate samples from the run C at the dilution of 6×10^{-1} prior to the treatment and after 5, 10, 15, 20, 30 and 40 minutes of ECP process. The error bars represent the standard deviation of two replicates.

Furthermore, both mortality and mobility assay were also performed for the samples obtained prior and at the end of the ECP treatments of the runs E and F. The results from the mortality assay showed that the viability of the nauplii after 24 hours of incubation in wastewater mixtures treated or not remained 100%. However, the results obtained from the mobility assay showed that the samples were not toxic either prior to the treatment or after it. The mobility of the nauplii was not inhibited more than 50%. However, a slight increase of the toxicity for both runs can be observed after the ECP process.
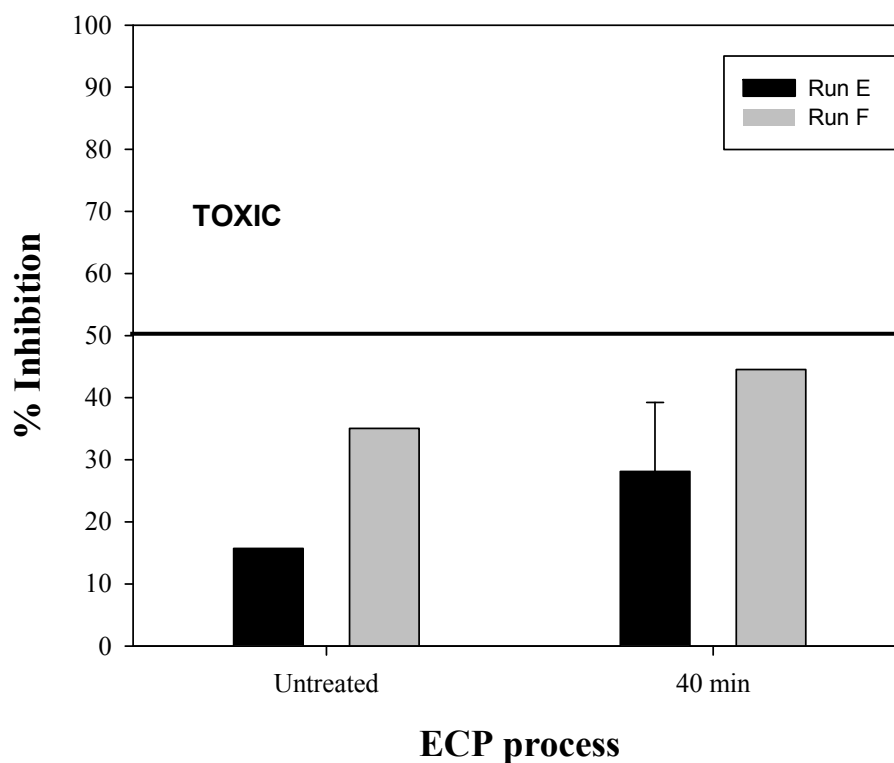
Figure 6.6 Percentage inhibition of the mobility of the *Artemia salina* nauplii in compost leachate samples at the dilution of 6 x 10^{-1} from run E and F prior and after the ECP treatment

6.4   Discussion

6.4.1 ECP with chloroplast bioassay

Storage time of the compost leachate samples on toxicity

Both treated and untreated wastewater samples are composed of various contaminants and the assessment of the toxicity is a challenging task. Various pre-treatments must be incorporated in order to minimise the toxicity by enhancing the particulate settling of possible existent contaminants. As proved from the results, low temperatures led to better settling of the compounds and as a result to the deterioration of the toxicity even if it ranged at low levels. In particular, the storage period of three days (fridge, 4 °C) had a positive effect on lowering the toxicity of the ECP treated leachate samples. Additionally, sequence degradations occurred and the levels of toxicity followed inconstant rates according to the obtained measurements with high degree of variance according to the error bars. These inconstant results might be due to non-homogeneous samples of wastewater.

Study the effect of sterilisation of compost leachate samples on toxicity

The sterilisation process performed in order to eliminate any potential effect of the existent microorganisms on the performance of the chloroplast assay. As a result, two batches of samples from the whole ECP process in various time intervals were obtained. The first one was treated without being sterilised and the second one was sterilised. The results did not show any particular difference between the batches. The toxicity ranged in low levels compared to the values achieved using atrazine.

However, an inconsistent pattern was observed comparing the samples of each batch during the ECP treatment. This can be explained by the degradation of the recalcitrant compounds occurred throughout the ECP process.

Finally, sterilisation proved less effective than expected since the chloroplast assay for both batches had slight differences. Consequently, the efficacy of the chloroplast assay is not dependent on the bacteria that exist on the treated or non- treated wastewater.

6.4.2 ECP with microbial assay

The specific microorganisms (*E. coli, P. aeruginosa* and *S. aureus)* were selected to be utilised to this assay since they exist in wastewater. Subsequently, they were applied as indicators of any alteration of the constitution of the samples prior and after the ECP treatment. The results showed that no modification of the composition of the samples to something more toxic was performed since the growth of the specific microorganisms was not affected in any sample.

6.4.3 ECP with invertebrates

The application of *Artemia salina* nauplii (instar stage II-III) as representative from the invertebrates was used for detecting potential risk of contamination in wastewater samples ECP-treated or not. Two endpoints of this organism were utilised for assessing the toxicity; the mortality of 8 nauplii and their mobility compared to controls. Furthermore, three runs (C, E and F) were applied and all the samples were tested for their toxicity prior and after the ECP process. More specifically, during the run C the assessment of the toxicity was also conducted during the ECP process.

The results from the aforementioned experiments showed that regardless of the survival of the nauplii, their mobility was inhibited. In particular, the results from run C indicated that the toxicity of the untreated samples after following an inconsistent pathway decreased after 40 minutes of process. However, the results from the runs E and F indicated that the untreated samples were not toxic but after the ECP process the toxicity increased.

6.5   Conclusions

The aim of the work discussed in this chapter was to assess the toxicity of untreated wastewater and then, to combine a wastewater remediation process, the ECP, with the already described in the previous chapters bioassays. More specifically, the chloroplast assay, the growth inhibition of *E. coli, P. aeruginosa* and *S. aureus*, and the mortality and mobility assay of *Artemia salina* nauplii were applied for evaluating the toxicity of leachate samples from the sewage treatment plant in Cranfield (UK) (run D) and compost leachate samples from Donarbon (Waterbridge, Cambridgeshire) (runs C, E and F). However, different toxicity assays were conducted for every run.

In particular, the assessment of the toxicity of wastewater samples of run B was conducted by utilising the chloroplast assay and the effect of low temperatures on the toxicity of the samples was also estimated. Furthermore, the chloroplast assay was applied in order to evaluate the effect of sterilisation on the toxicity of compost leachate samples from run C, and the mortality and the inhibition of mobility of *Artemia salina* nauplii in the non-sterilized samples were also determined. Wastewater samples from run D were assessed for their toxicity using the microbial assay; the growth inhibition of *E. coli, P. aeruginosa* and *S. aureus* in toxic environment. Then, the toxicity level of the samples from the run E was estimated using the microbial assay, the mortality and the inhibition of the mobility of *Artemia salina* nauplii assay. Finally, the toxicity of the wastewater samples from run F was determined using the effect on mortality and mobility of *Artemia salina* nauplii.

The results obtained from the run B showed that the toxicity of the samples ranged in low levels but with high variation among the samples in some cases according to the error bars. Regarding the results obtained from the toxicity assays from the run C, it was obvious that the samples were not toxic either prior or after the treatment according to the chloroplast assay and the mortality assay of *Artemia salina* nauplii. Furthermore, the chloroplast assay was not impaired by the existence of bacteria in the samples. However, the mobility of the nauplii was inhibited at approximately 50% in the untreated samples but during the ECP treatment the inhibition increased. At the end of the process, though, the mobility of the nauplii reached approximately the level of the control samples.

Regarding the results from the run D, the applied microorganisms grew normally regardless the presence of the saturated disc with wastewater samples; treated or not.

As far as the run E, the results from all the applied bioassays indicated that the untreated wastewater samples and the after-ECP-treated samples were not toxic. However, in some cases the toxicity increased at the end of the ECP treatment which caused by the presence of ferrous and ferric ions that are toxic to *Artemia salina* nauplii. Another reason for this raise of toxicity level is the degradation of the recalcitrant compounds which resulted in the production of smaller compounds which are usually more toxic.

Finally the results obtained from the run F showed that the toxicity of the samples was low since all the inserted nauplii in the samples remained alive after 24 hours of incubation. However, it was observed that the inhibition of the mobility of the nauplii was increased after the ECP treatment but remained at low level.

In conclusion, the results obtained from the several runs of ECP treatment in wastewater samples regarding their toxicity showed that the results of each assay verified the results obtained from the other assay applied on the specific samples. The application of more toxicity assays on wastewater samples treated or not, enhances the reliability of the results and their sensitivity to a great range of contaminants. Consequently, the application of a battery of assays is an imperative demand since the mixture of contaminants may vary in wastewater samples.

Chapter 7   General Discussion, Conclusions and Future Recommendations

7.1   Discussion

The general increase of the wastewater level produced by industries, housing estates and commercial activities has raised issues of further treatment and assessment of the toxicity. Since the presence of dangerous compounds in wastewater has been verified, the awareness of potential contamination of the surface water including lakes and rivers has been increased and stricter regulations have been established. As a result, the demand for applying effective treatment and monitoring facilities for wastewater is higher these days.

Wastewater remediation processes are often broadly divided into biological or physico-chemical processes. Very often, combinations of these two are applied to provide an optimum treatment regime for a particular waste stream. Biological processes, including activated sludge or rotating biological contactors are of high cost and may also demand further steps for removing heavy metals and inorganic compounds. Furthermore, they are not easily portable and sometimes treatment via these methods is often a very slow process. Regarding the physical or chemical treatments, these usually incorporate techniques including ozonation, chlorination or filtration, which are also rarely cost-efficient nor sufficiently effective. The ECP process offers an effective treatment of wastewater of a similar standard of remediation to the biological treatments, but it can be portable and needs little pre-treatment (Paton *et al.,* 2009). Additionally, the ECP method has been applied for treating wastewater samples and has effectively reduced BOD and COD at a level of 36% and 52% respectively (Paton *et al.,* 2009). This process has been demonstrated over the last few years to be an effective method to degrade large molecules which are usually recalcitrant, insoluble and stable in the environment to smaller molecules which are more mobile, more soluble but frequently more toxic.

In Chapter 2, some parameters of the working protocol of the ECP were optimised in order to achieve a more efficient way of treating complex mixtures. Storage at low temperatures

proved the most efficient step to the working protocol in order to further examine the toxicity of the samples. The particulate settling of the contaminants occurred during the storage period at 4°C led to clear samples of water. Regarding the pH, two values of pH 4 and pH 7 were tested for their effect on colour removal of the samples using a spectrophotometer. During the ECP process, the pH of the treated effluent must be lowered to pH 4 with a strong acid such as H_2SO_4, which also raises the conductivity, increasing the number of ions to be produced. In addition, the solubility of the iron (as ferrous ions) is enhanced at lower pH values, improving its release from the anode and, hence, the performance of the ECP process (Arienzo *et al,* 2001b). After treatment had been completed, the samples were examined for the pH effect on their turbidity, since this value of the pH is an important factor for the following toxicity analysis. Although more turbid solutions were obtained, the demand of re-adjusting the pH to approximately 7 was essential. Subsequently, a possible combination of pH adjustment with storage at low temperatures proved necessary.

During 2010, a larger-scale ECP prototype was designed and constructed by a project collaborator, Environmental Oxygen Ltd (Leicester, UK). The laboratory scale ECP unit circulated the wastewater samples for 40 minutes. The newly developed unit (Colour plate 7.1) has a flow-through system during which the wastewater passes through three reactors, sequentially (Colour plate 7.2). Each of these reactors has the same basic construction as the laboratory unit and the treated water ends in a large storage tank. The laboratory scale design did not allow the effluent to come in full contact with the electrodes, which resulted in reduced efficiency of the treatment. In contrast, the newly implemented design allows the wastewater to come in full contact with the electrodes since now the reactor is in vertical position.

Colour plate 7.1 The completed unit.

Colour plate 7.2 The reactors.

However, the wastewater, in its untreated and treated forms, has a very complex constitution with possible high toxicity levels which must be assessed. Greater demand for rapid and reliable monitoring methods has been observed over the last few years for detecting any potential hazard for the environment and particularly the exposed living organisms. However, most of the commercially available devices are using a live organism from the categories of plants, algae, microorganisms and invertebrates in order to assess the toxicity of contaminated samples. Some of these include LuminoTox (Lab Bell Inc.) which applies algae (*Chlorella vulgaris*), Microtox® which uses *V. Fischeri* and the *Rapidtoxkit* (Biohidrica®, Chile) which utilises the crustacean *Thamnocephalus platuyurus*. In addition, toxicity assays involving higher organisms have also been applied but in this study they were not further investigated since they require special facilities, ethical approval and in some cases, a licence.

The work described in chapters 3, 4 and 5 is related to toxicity assays using plant cells (Chapter 3), microorganisms (Chapter 4) and invertebrates (Chapter 5) which were developed in the laboratory and applied in order to assess the toxicity of specific compounds which are usually present in water (groundwater, rivers, lakes, wastewater, etc). The summary of the results and their sensitivity are displayed in Table 7.1.

First of all, a plant cell based assay was applied which used chloroplasts extracted from spinach as described in chapter 3 in detail. The results indicated that the detection of herbicides and the evaluation of their toxic effect can be performed using this assay with high efficiency. Furthermore, the tested heavy metal ions showed a moderate response but there was not sensitivity for the specific organic and inorganic compounds tested.

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Table 7.1 Summary of the results from Chapters 2, 3, 4, 5 and 6.

	Chapter 3 Plant cells	**Chapter 4 Microbial assays**		**Chapter 5 Invertebrate assays**	
	Chloroplast assay	*E. coli,* *P. aeruginosa* *S. aureus*	*V.fischeri*	Mortality of Artemia salina nauplii	Mobility of Artemia salina nauplii
Atrazine	✔	✘	—	✘	?
Copper ions	?	✘	—	✔	✔
Phenol	✘	✔	✔	✔	✔
Sodium hypochlorite	✘	✔	✔	✔	✔

✔ : Detected from the specific assay

✘ : No detection from the specific assay

? : Ambiguous response

— : Not analysed

Then, the toxic effect of the same toxic compounds was estimated by measuring the growth inhibition of *E. coli, P. aeruginosa* and *S. aureus* and the bioluminescence inhibition emitted by *V.fischeri* (Chapter 4). The first assay, showed partial sensitivity only to phenol and sodium hypochlorite and to the most concentrated applied dilutions. Phenol and sodium hypochlorite were only applied to the second assay since the *V.fischeri* cultures were problematic and stopped emitting light. The results indicated that the samples were not toxic to *V. fischeri* at the specific concentrations. The application of the three microorganisms that naturally occur in wastewater treatment was useful as an indicator of the change in the composition of the wastewater samples (Chapter 6). Regarding *V.fischeri*, many advanced technologies have been developed using either freeze-dried bacteria (Microtox®) or continuously produced *V.fischeri* cells Cymtox (Modern Water, UK) which monitors any possible contaminant existent in the water. However, freshly prepared cells are possibly unreliable for estimating the toxicity since their activity to emit light can be depressed any time and consequently, the reproducibility and reliability of the results to be eliminated.

Furthermore, the application of two endpoints of *Artemia salina*, mortality and mobility, in order to assess the toxicity level of aqueous solutions was performed (Chapter 5). The selection of *Artemia salina* species happened due to the availability in Cranfield Health laboratory and ethical approval or a special licence was not required. The same compounds as before were initially tested.

The results from the mortality assay showed the expected sigmoid pattern to all the applied compounds apart from atrazine. Regarding the results from the mortality assay, they were higher than those often reported in the literature. This is due to the low number of nauplii applied in these assays (4 or 8) since the further process, probit analysis, is based on proportions.

Later, a novel device was implemented for monitoring the mobility of *Artemia salina* nauplii as an indication of toxic effects of various harmful compounds. Videos were recorded and analysed using digital image processing for tracking the nauplii inside the toxic solution with high accuracy. The parameters which affected the process and the device, were optimised by firstly specifying the most appropriate temperature range which was found to be 15-25°C, at which the nauplii retained their mobility for 24 hours. This means that under standard laboratory conditions, temperature control is likely to be unnecessary, which should minimise instrument cost.

The clarity of the samples remains a concern, since it was seen to restrict the ability of the system to follow the movement of the nauplii in more turbid solutions. Although undesirable, filtration or settling (especially under refrigeration) has been seen to reduce this problem, albeit adding to the assay complexity.

The incubation period was successfully reduced to two thirds, allowing measurements to be performed after 16 hours without significantly affecting the results, compared to the initial assay, which was carried out after 24 hours. Nevertheless, most experiments used a 24 hour incubation time, since it was more convenient for the performance of the experiments.

The nauplii population was varied between 4 and 8 nauplii in each cell, to assess the number of organisms on the accuracy of the test. The results obtained, indicated that there was no significant difference, according to ANOVA analysis. However, slightly higher sensitivity was obtained when 4 nauplii were used, but at the expense of slightly higher error than when 8 nauplii were inserted in the cuvettes.

Various contaminants were assessed successfully for their toxicity including heavy metal ions, pesticides, pharmaceuticals, mixtures of contaminants and finally wastewater samples (Chapter 6). More specifically, the toxicity of heavy metal ions it was detected below the level indicated by European regulations. Additionally, among the pollutants tested, there also were some that are usually proposed by EPA-ETV in order new technologies to be verified such as aldicarb, colchicine and thallium sulphate. These compounds showed that the threshold toxicities obtained by the novel device were comparable to ones by Microtox® for the same compounds and significantly lower than the human lethal doses. As a result, the proposed system offers a low-cost alternative to toxicity monitoring.

Furthermore, a protocol for using this device in order to identify potential toxic solutions using swarm of nauplii was also developed. The insertion of random and un-quantified number of nauplii facilitated the process since the counting of specific amount of nauplii is a laborious procedure, which is prone to possible errors. Finally, an uncounted swarm of nauplii was inserted to mixtures of contaminants and the toxicity was successfully detected and estimated with higher sensitivity than occurred when 4 nauplii were used for the same compound (ferrous ion solutions).

Commercial competition in the market is relatively low regarding the newly-developed device and its concept. There is a piece of commercially-available software sold (Ethovision,

Noldus Information Technology, Netherlands) which measures the movement of the tested organisms and their behaviour to specific drugs. However, this software package does not provide the ability to estimate the IC_{50} values and cannot conclude if a solution is toxic or not. In the published study regarding *Artemia salina* nauplii and Ethovision (Venkateswara *et al.*, 2007), the researchers applied *Artemia salina* on the already estimated by the mortality assay EC_{50} values of specific compounds. Then, they compared their results by estimating the distance that has been covered by the nauplii in specific time with the already estimated LC_{50} values. The application of the newly developed device of this study is a tool for estimating the IC_{50} values and identifying if a sample of unknown constitution is toxic or not with high sensitivity and in low cost. However, the development of a prototype is a demand in order the human error to be minimised. An advanced battery and a sealed chamber are required in order to increase the accuracy of the measurements.

In the last chapter (6), the toxicity assays applied in the previous chapters (3, 4 and 5) and tested for single contaminants and occasionally to mixtures, were applied in wastewater samples. The assessment of the toxicity of untreated wastewater and then, the combination of the ECP with those bioassays were performed. More specifically, the chloroplast assay, the growth inhibition of *E. coli, P. aeruginosa* and *S. aureus*, and the mortality and mobility assay of *Artemia salina* nauplii were applied for evaluating the toxicity of leachate samples from the sewage treatment plant in Cranfield (UK) (run D) and compost leachate samples from Donarbon (Waterbridge, Cambridgeshire) (runs B, C, E and F). However, different toxicity assays or combinations of those were conducted for every run.

The obtained results showed an agreement among the applied bioassays when two or more were performed. Furthermore, the applications of assays that are sensitive to different groups of contaminants indicated the higher sensitivity when applied as set of assays. However, the initial hypothesis regarding the high level of toxicity of the samples prior to the ECP treatment and the decrease after this was not confirmed since the samples were not initially toxic according to the obtained results. The only partial exception was with the samples from the run C. In this case, the mobility of *Artemia salina* naupliin was inhibited approximately 50% and after 40 minutes of ECP treatment the inhibition decreased to approximately 20%. This result means that the samples prior to the treatment were partially toxic and after the ECP process the wastewater samples became even less toxic. However, in some cases the toxicity slightly increased after the ECP treatment. This was possibly due to the presence of ferrous and ferric ions which are especially toxic to *Artemia salina* nauplii as proved in

chapter 5 (Section 5.3.6). Another reason for this increase of toxicity level is the degradation of the recalcitrant compounds which resulted in the production of smaller compounds which are usually more toxic. However, this needs to be confirmed with treatment of more toxic samples.

To summarise, wastewater samples were treated using the ECP process and the toxicity was assessed with the developed assays which were initially tested to single contaminants. Furthermore, a newly developed device was designed, constructed, optimised and finally applied to toxic aqueous solutions. The obtained results indicated that their combination in detecting toxicity in complex solutions is necessary in order to increase the reliability and the sensitivity of the results. Finally, the low cost of the assays enhanced their application.
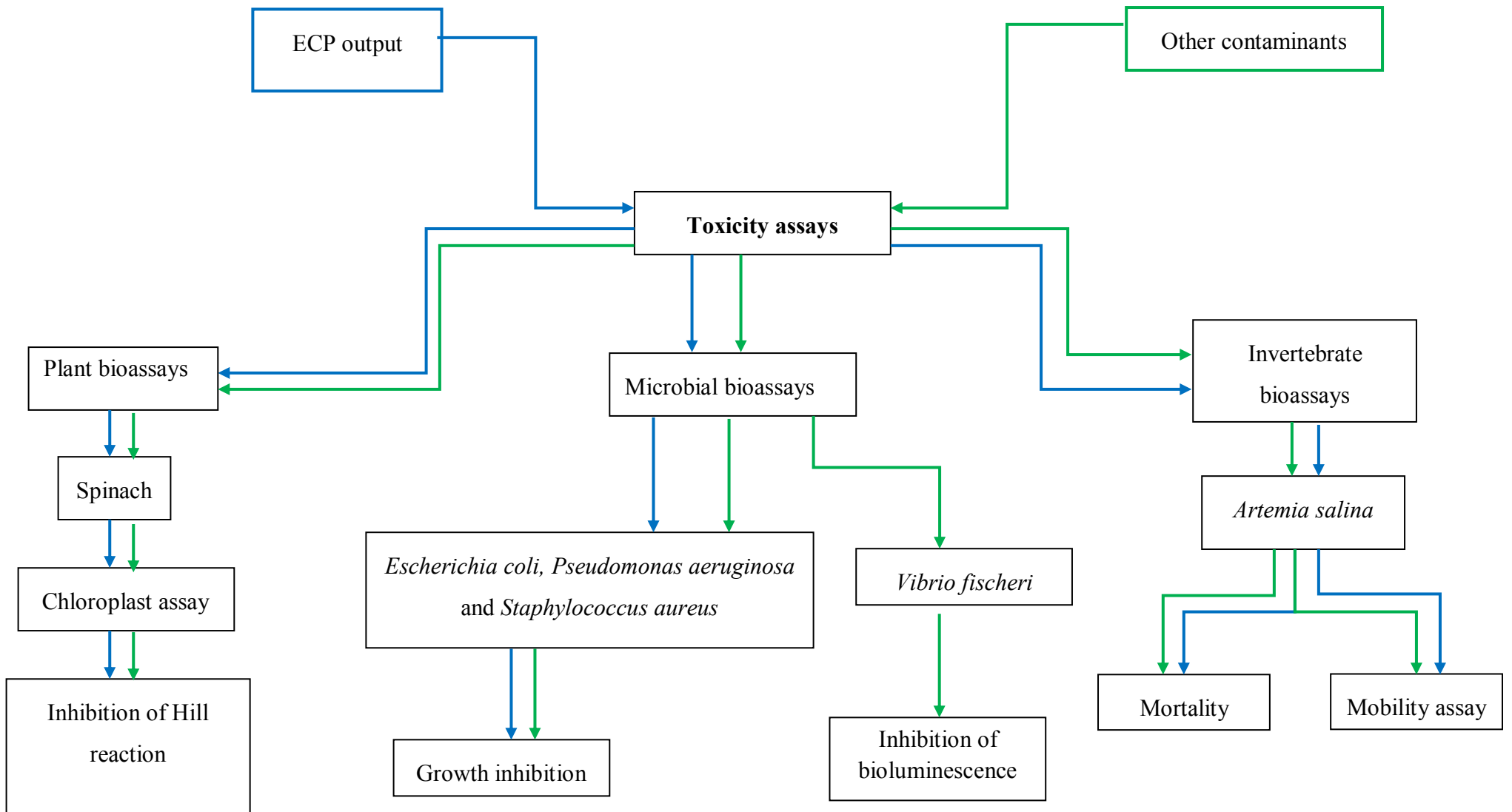
Figure 7.1 Summary flow diagram.

7.2   Conclusions

➢ To optimise the working protocol of the ECP for treating wastewater. Low temperature storage was applied to ECP treated samples. This treatment enhanced the particulate settling of the existing compounds and consequently, the turbidity level was minimised (Chapter 2). The obtained level of clarity was helpful for assessing the toxicity by performing the newly developed device using *Artemia salina* nauplii (Chapter 5). The pH of the ECP treated samples should be re-established to approximately 7 in order the toxicity assays to be performed. The most appropriate H_2O_2 injection method was not identified due to problems faced.

➢ To apply toxicity assays based to different organisms (plants, microorganisms and invertebrates) for assessing the toxicity of single contaminants and mixtures. Toxicity assays using plant cells (chapter 3) - the chloroplast assay-, microorganisms (chapter 4) - growth inhibition of *E. coli, P. aeruginosa* and *S. aureus* and the bioluminescence inhibition emitted by *V.fischeri* - and invertebrates (chapter 5) – mortality and mobility of *Artemia salina* nauplii showed different sensitivity to the same contaminants. Combination of those should be the most ideal solution for detecting toxicity in complex solutions.

➢ To develop and optimise novel assays for detecting potential toxic aqueous solutions. A novel device has been developed which tracks successfully the mobility of *Artemia salina* nauplii in contaminated aqueous solutions (chapter 5). The optimum conditions of temperature (15-25°C), the effect of turbidity and the possibility of minimisation of the exposure time to 16 hours were determined. This system estimates successfully the average speed per nauplius, which proved to be dependent to the toxicity level of tested samples. The toxicity of heavy metal ions was efficiently detected below the level indicated by European regulations. In addition, the effectiveness and suitability of the device was also verified when compounds proposed by the Environmental Protection Agency were applied. In particular, aldicarb, colchicine and thallium sulphate showed that the threshold toxicities obtained by the novel device were

comparable to ones by Microtox and significantly lower than the human lethal doses. Furthermore, the accuracy of the device is higher when a random and un-quantified number of nauplii are applied. As a result, the proposed system offers a low-cost alternative to toxicity monitoring.

➢ <u>To optimise the working protocol for estimating the toxicity of wastewater samples following the ECP process.</u> Various combinations of the chloroplast assay, the growth inhibition of *E. coli, P. aeruginosa* and *S. aureus* and the mortality and mobility of *Artemia salina* nauplii were applied in order to estimate the level of toxicity for the wastewater samples of various runs (Chapter 6). The results from the sets of assays applied showed that the level of toxicity prior and after the ECP treatment ranged in low levels. However, during the ECP process an inconsistent pattern of toxicity was observed which was explained by the degradation of the recalcitrant compounds exist in wastewater. Finally, the application of a battery of assays is an imperative demand since the variety of contaminants is high in wastewater samples. An optimised methodology for estimating the toxicity of wastewater samples following the ECP process using a battery of assays was concluded and presented in Figure 7.2.
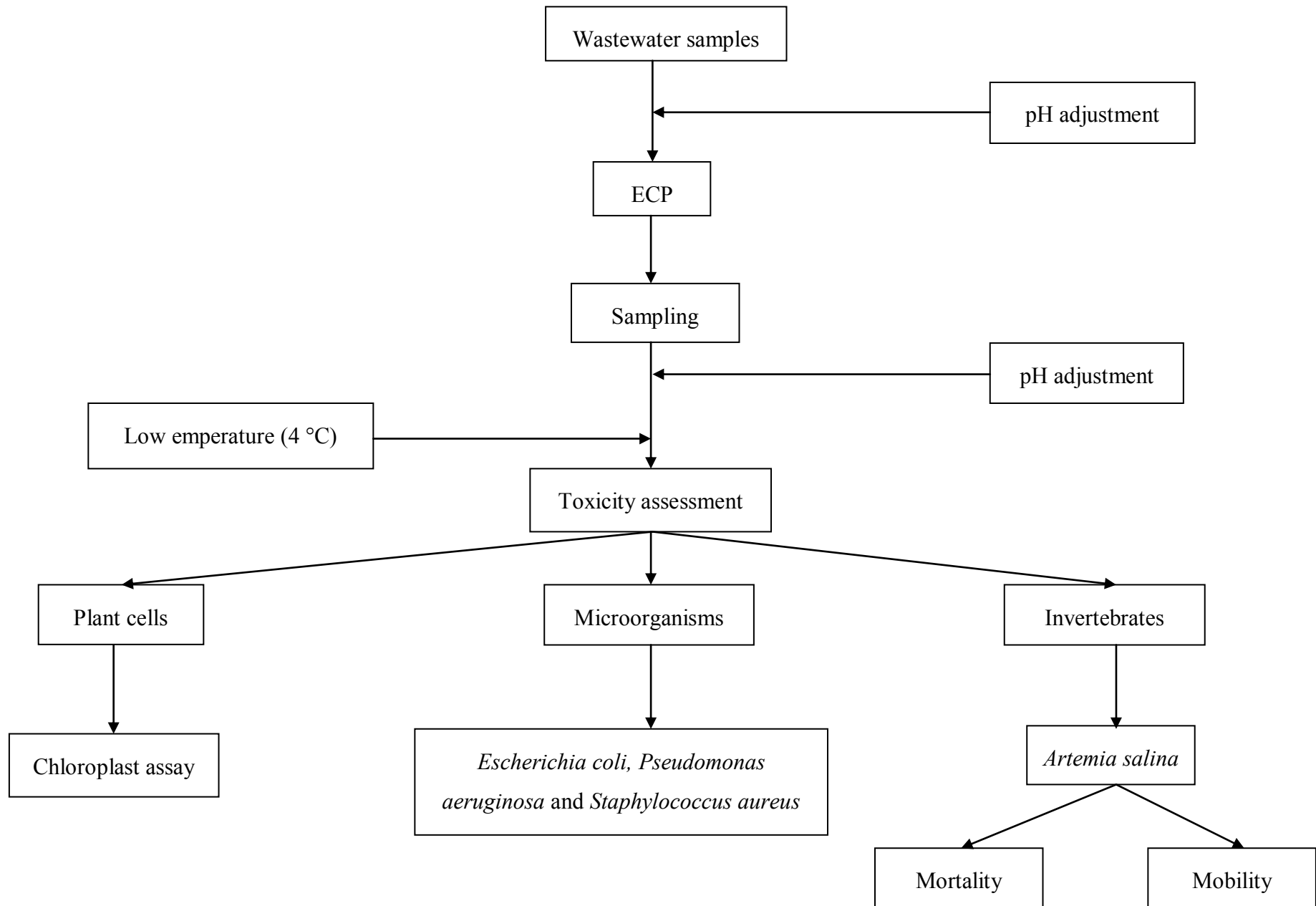
Figure 7.2 Concluded methodology.

7.3   Future recommendations

More wastewater samples from various sources and of higher toxicity level would be interesting to be treated using ECP and assessed for their toxicity. Chemical analysis prior to and following the ECP treatment using analytical techniques including Gas Chromatography/ Mass Spectrometry (GC/MS) or High Performance Liquid Chromatography would be of high interest. Furthermore, chemical and toxicity analysis of the sludge produced after the ECP treatment would be valuable to be performed before the discharge to the environment. Further chemical analysis of the untreated and ECP-treated samples for identifying the hydroxyl radical attacking pathways would be of high concern.

Regarding the newly developed device, a prototype must be constructed in order the error, including light reflections and battery yield, to be minimised. Furthermore, an automatic way for recording the samples should be incorporated in the prototype in order the human error to be eliminated.

Furthermore, regarding the working protocol of this device, more experiments as far as the minimisation of the incubation period to less than 16 hours would be useful to be tested in order to establish a quick response bioassay. More life stages of *Artemia salina* nauplii can be applied for evaluating the most sensitive but also stable for the respective exposure period. In addition, more contaminants and mixtures of them would be also useful to be tested. Moreover, a comparative study between this device and an already existed in the market device for the same contaminants would also be valuable to be investigated. This device can also be applied with other organisms such as Daphnia magna for assessing the toxicity of aqueous solutions.

Finally, a possible integration of the ECP process to a set of toxicity assays in order the toxicity of the treated wastewater to be assessed straight after the process would be of high interest.

Chapter 8 References

Al-Kdasi, A., Idris, A., Saed, K. and Guan, C. T. (2004), "Treatment of textile wastewater by advanced oxidation processes-a review", *Global Nest: The Int. J.,* vol. 6, no. 3, pp. 226-234.

Al-Mahmoud, M. S., Alali, F. Q., Tawaha, K. and Qasaymeh, R. M. (2006), "Phytochemical study and cytotoxicity evaluation of Colchicum stevenii Kunth (Colchicaceae): A Jordanian meadow saffron", *Natural Product Research,* vol. 20, no. 2, pp. 153-160.

Andrews, J. M. (2005), "BSAC standardized disc susceptibility testing method (version 4)", *Journal of Antimicrobial Chemotherapy,* vol. 56, no. 1, pp. 60-76.

Arienzo, M., Adamo, P., Chiarenzelli, J., Bianco, M. R. and De Martino, A. (2002), "Retention of arsenic on hydrous ferric oxides generated by electrochemical peroxidation", *Chemosphere,* vol. 48, no. 10, pp. 1009-1018.

Arienzo, M., Chiarenzelli, J. and Scrudato, R. (2001a), "Removal of arsenic in aqueous solution by electrochemical Peroxidation", *Fresenius Environmental Bulletin,* vol. 10, no. 9, pp. 731-735.

Arienzo, M., Chiarenzelli, J., Scrudato, R., Pagano, J., Falanga, L. and Connor, B. (2001b), "Iron-mediated reactions of polychlorinated biphenyls in electrochemical peroxidation process (ECP)", *Chemosphere,* vol. 44, no. 6, pp. 1339-1346.

Armenante P.M. (1999), "Precipitation of heavy metals from wastewater", New Jersey Institute of Technology

Arnold, S. M., Hickey, W. J. and Harris, R. F. (1995), "Degradation of atrazine by Fenton's reagent: Condition optimization and product quantification", *Environmental Science and Technology,* vol. 29, no. 8, pp. 2083-2089.

Atwater J.W., Jasper S., Mavinic D.S., and Koch F.A., (1983), "Experiments using *Daphnia* to measure landfill leachate toxicity", *Water Resources*, vol.17, 1855-1861.

Babich, H. and Borenfreund, E. (1991), "Cytotoxicity and genotoxicity assays with cultured fish cells: A review", *Toxicology in Vitro,* vol. 5, no. 1, pp. 91-100.

Bagshaw, J. C., Rafiee, P., Matthews, C. O. and MacRae, T. H. (1986), "Cadmium and zinc reversibly arrest development of Artemia larvae", *Bulletin of environmental contamination and toxicology,* vol. 37, no. 2, pp. 289-296.

Barahona M.V. and Sánchez-Fortún S. (1996), "Comparative Sensitivity of Three Age Classes of Artemia salina Larvae to Several Phenolic Compounds", *Bulletin of Environmental Contamination and Toxicology*, **56**(2), 271-278.

Barb, W. G., Baxendale, J. H., George, P. and Hargrave, K. R. (1951), "Reactions of ferrous and ferric ions with hydrogen peroxide. Part I. - The ferrous ion reaction", *Transactions of the Faraday Society,* vol. 47, pp. 462-500.

Barton, P. (2005), "Electrochemical Peroxidation of Compost Leachate", MSc thesis submitted to Cranfield University, UK.

Baumstark-Khan, C., Hellweg, C. E., Scherer, K. and Horneck, G. (1999), "Mammalian cells as biomonitors of UV-exposure", *Analytica Chimica Acta,* vol. 387, no. 3, pp. 281-287.

Berg, J.M., Tymoczko, J.L. and Stryer, L. (2002), "Biochemistry", 5th Edition, W.H. Freeman and Company, New York, pp: 527-534.

Bernard, C., Guido, P., Colin, J. and Anne, L. D. (1996), "Estimation of the hazard of landfllls through toxicity testing of leachates. I. Determination of leachate toxicity with a battery of acute tests", *Chemosphere,* vol. 33, no. 11, pp. 2303-2320.

Bitton, G. (1983), "Bacterial and biochemical tests for assessing chemical toxicity in the aquatic environment: a review", *Critical Reviews in Environmental Control,* vol. 13, no. 1, pp. 51-67.

Bortolotto, T., Bertoldo, J. B., Silveira, F. Z. d., Defaveri, T. M., Silvano, J. and Pich, C. T. (2009), "Evaluation of the toxic and genotoxic potential of landfill leachates using bioassays", *Environmental toxicology and pharmacology,* vol. 28, no. 2, pp. 288-293.

Brillas, E. and Casado, J. (2002), "Aniline degradation by Electro-Fenton® and peroxi-coagulation processes using a flow reactor for wastewater treatment", *Chemosphere,* vol. 47, no. 3, pp. 241-248.

Brix, K. V., Gerdes, R. M., Adams, W. J. and Grosell, M. (2006), "Effects of copper, cadmium, and zinc on the hatching success of brine shrimp (Artemia franciscana)", *Archives of Environmental Contamination and Toxicology,* vol. 51, no. 4, pp. 580-583.

Broers, C.A.M, and Lappalainen J., (2004), "New developments in the bioluminescence assay", *Courrier du Savoir,* no 05, pp. 107-110.

Brown, B. and Ahsanullah, M. (1971), "Effect of heavy metals on mortality and growth", *Marine pollution bulletin,* vol. 2, no. 12, pp. 182-187.

BS EN ISO 11348-1:1999, "Water quality – Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) ", British Standard, European Standard, International Organization for Standardization, 15-Mar-1999, 26 pages

BSI PAS 100. (2005), "Specification for composted materials". British Standards Institution, Publicly Available Specification.

Buffin, B.P., (1999), "Removal of Heavy Metals from Water: An Environmentally Significant Atomic Absorption Spectrometry Experiment", *J. Chem. Ed., vol. 76,* pp. 1678.

Calabrese, E.J. and P.T. Kostecki. (1989), "Petroleum Contaminated Soil", Remedation Technologies, Environmental Fate, Risk Assessment, Analytical Methodologies, vol. 2, Lewis Publishers Inc., Chelsea.

Cameron R.D., Koch F.A., (1980), "Toxicity of landfill leachates", *J. Water Pollut. Control Fed.,* vol. 52, pp. 760-769

Campanella, L., Colapicchioni, C., Favero, G., Sammartino, M. P. and Tomassetti, M. (1996), "Organophosphorus pesticide (Paraoxon) analysis using solid state sensors", *Sensors and Actuators B: Chemical,* vol. 33, no. 1-3, pp. 25-33.

Carballo, J. L., Hernández-Inda, Z. L., Pérez, P. and García-Grávalos, M. D. (2002), "A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products.", *BMC biotechnology [electronic resource],* vol. 2, no. 1, pp. 17.

Cheung K.C., Chu L.M. and Wong M.H., (1993), "Toxic effect of landfill leachate on microalgae", *Water Air Soil Pollut.*, vol. 69, pp. 337-349.

Chiarenzelli, J. R., Scrudato, R. J., Wunderlich, M. L. and Pagano, J. J. (2001), "Combined steam distillation and electrochemical peroxidatiol (ECP) treatment of river sediment contaminated by PCBs", *Chemosphere,* vol. 45, no. 8, pp. 1159-1165.

Costa L.G. (2001), "Toxic effects of pesticides", In: Casarett and Doull's Toxicology the Basic Science of Poisons. Mc Graw-Hill, Medical Publishing Division, Sixth Edition, pp. 883-884

Council Directive 91/271/EC (1991), "Concerning urban waste-water treatment". Official Journal of the European Communities.

Couture, P., Blaise, C., Cluis, D. and Bastien, C. (1989), "Zirconium toxicity assessment using bacteria, algae and fish assays", *Water, Air, and Soil Pollution,* vol. 47, no. 1-2, pp. 87-100.

Cox C. (1992), "Aldicarb", *Journal of Pesticide*, Reform 12

Dalley R (2001), "Copper briefing paper", Water UK, London

Davì, M. L. and Gnudi, F. (1999), "Phenolic compounds in surface water", *Water research,* vol. 33, no. 14, pp. 3213-3219.

De Laat, J. and Gallard, H. (1999), "Catalytic decomposition of hydrogen peroxide by Fe(III) in homogeneous aqueous solution: Mechanism and kinetic modeling", *Environmental Science and Technology,* vol. 33, no. 16, pp. 2726-2732.

Decision No 0455/2001/EC (2001), "Establishing the list of priority substances in the field of water policy and amending Directive 2000/60/EC", *Official Journal of the European Communities,* December 15, 2001.

Directive 2000/60/EC (2000), "Establishing a framework for community action in the field of water policy", *Official Journal of the European Communities*, December 22, 2000.

Directive 2008/105/EC (2008), "Environmental quality standards in the field of water policy", *Official Journal of the European Communities*, December 16, 2008.

Dojmi Di Delupis, G. and Rotondo, V. (1988), "Phototaxis in aquatic invertebrates: Possible use in ecotoxicity test", *Ecotoxicology and environmental safety,* vol. 16, no. 3, pp. 189-193.

Duncan F. (2005), "Disc diffusion susceptibility methods", Applied microbiology lab, pp. 1-6

Eaton D.L. and Klaasen C. C. (2001), "Principles of toxicology", In: Casarett and Doull's Toxicology The Basic Science of Poisons. Mc Graw-Hill, Medical Publishing Division, Sixth Edition, pp. 11-14

Echobichon D.J. (2001b), "Toxic effect of pesticides", In: Klaassen CD (ed): Handbook of pesticide toxicology. San Diego: Academic Press, 2001b, pp. 1087-1106.

Eilbeck, W.J., and Mattock, G. (1987), **"**Chemical processes in waste water treatment", edited by Ellis Horwood, New York, pp 165-200.

El-Alawi, Y. S., McConkey, B. J., George Dixon, D. and Greenberg, B. M. (2002), "Measurement of short- and long-term toxicity of polycyclic aromatic hydrocarbons using luminescent bacteria", *Ecotoxicology and Environmental Safety,* vol. 51, no. 1, pp. 12-21.

Ernst W.R., Hennigar P., Doe K., Wade S., Julien G., (1994), "Characterization of the chemical constituents and toxicity to aquatic organisms of a municipal landfill leachate", *Water Poll. Res. J. Canada*, vol. 29, pp. 89-101.

Evans, M. R., Jordinson, G. M., Rawson, D. M. and Rogerson, J. G. (1998), "Biosensors for the measurement of toxicity of wastewaters to activated sludge", *Pesticide Science,* vol. 54, no. 4, pp. 447-452.

Farré, M. and Barceló, D. (2003), "Toxicity testing of wastewater and sewage sludge by biosensors, bioassays and chemical analysis", *TrAC - Trends in Analytical Chemistry,* vol. 22, no. 5, pp. 299-310.

Farré, M., Pasini, O., Carmen Alonso, M., Castillo, M. and Barceló, D., (2001), "Toxicity assessment of organic pollution in wastewaters using a bacterial biosensor", *Analytica Chimica Acta,* vol. 426, no. 2, pp. 155-165.

Ferrari, B., Radetski, C. M., Veber, A., and Ferard, J., (1999), "Ecotoxicological assessment of solid wastes: A combined liquid- and solid-phase testing approach using a battery of bioassays and biomarkers", *Environmental Toxicology and Chemistry,* vol. 18, no. 6, pp. 1195-1202.

Freeman, B. (2002), "Electrochemical Peroxidation of Landfill Leachate", MSc thesis submitted to Cranfield University, UK.

Gajbhiye S.N. and Hirota R. (1990), "Toxicity of heavy metals to brine shrimp Artemia", *Journal of the Indian fisheries Association*, vol. 20, pp. 43-50

Giannis, A., Kalaitzakis, M. and Diamadopoulos, E. (2007), "Electrochemical treatment of olive mill wastewater", *Journal of Chemical Technology and Biotechnology,* vol. 82, no. 7, pp. 663-671.

Guerra, R. (2001), "Ecotoxicological and chemical evaluation of phenolic compounds in industrial effluents", *Chemosphere,* vol. 44, no. 8, pp. 1737-1747.

Gutiérrez, M. C., Pepió, M., Crespi, M. and Mayor, N. (2001), "Control factors in the electrochemical oxidation of reactive dyes", *Coloration Technology,* vol. 117, no. 6, pp. 356-361.

Haber, F. and Weiss, J. (1934). "The catalytic decomposition of hydrogen peroxide by iron salts", *Proc. Roy.Soc.London A.*, vol. 147, pp. 332–351.

Hammami, S., Oturan, N., Bellakhal, N., Dachraoui, M. and Oturan, M. A. (2007), "Oxidative degradation of direct orange 61 by electro-Fenton process using a carbon felt electrode: Application of the experimental design methodology", *Journal of Electroanalytical Chemistry,* vol. 610, no. 1, pp. 75-84.

Harwig J and Scott P. M. (1971), "Brine Shrimp (*Artemia salina L.*) Larvae as a Screening System for Fungal Toxins", *Applied and Environmental Microbiology,* vol. 21, no. 6, pp. 1011-1016.

Hawksley R. (2003), "Electrochemical Peroxidation of Contaminated Water", MSc thesis submitted to Cranfield University, UK.

Hernando, M.D., Fernández-Alba, A.R., Tauler, R. & Barceló, D., (2005), "Toxicity assays applied to wastewater treatment", *Talanta,* vol. 65, no. 2 SPEC. ISS., pp. 358-366.

Hu A. (2002), "Looks can be deceiving: the case of Escherichia coli", *Journal of Young Investigators,* vol. 6, no. 5.

Huang, C. P., Dong, C. and Tang, Z. (1993), "Advanced chemical oxidation: Its present role and potential future in hazardous waste treatment", *Waste Management,* vol. 13, no. 5-7, pp. 361-377.

Huang, Y., Chen, C., Huang, G. and Chou, S. S. (2001), "Comparison of a novel electro-Fenton method with Fenton's reagent in treating a highly contaminated wastewater", *Water Science and Technology,* vol. 43, no. 2, pp. 17-24.

Huang, Y., Chou, S., Perng, M., Huang, G.. and Cheng, S., (1999), "Case study on the bioeffluent of petrochemical wastewater by electro-Fenton method", *Water Science and Technology,* vol 39, no 10-11, pp 145–149.

Huang, Y., Huang, Y., Chang, P. and Chen, C., (2008), "Comparative study of oxidation of dye-Reactive Black B by different advanced oxidation processes: Fenton, electro-Fenton and photo-Fenton", *Journal of Hazardous Materials,* vol. 154, no. 1-3, pp. 655-662.

Isidori, M., Lavorgna, M., Nardelli, A. and Parrella, A. (2003), "Toxicity identification evaluation of leachates from municipal solid waste landfills: A multispecies approach", *Chemosphere,* vol. 52, no. 1, pp. 85-94.

ISO, Water Quality: "Test for Inhibition of Oxygen Consumption by Activated Sludge", ISO 8192, Part A, International Standardization Organization, Geneva, 1986, pp 1-10.

Jean G., Fruget J.F., (1994), "Comparison of ecotoxicological and physico-chemical data by use of multivariate analyses and graphical displays", *Chemosphere*, vol. 28, 2249-2267.

Jenkins, J. (2005), "The Humanure Handbook", Chelsea Green Publishing, 3rd Edition, USA, pp: 41-43

Jury, S. (2004), "Electrochemical Peroxidation of Landfill Leachate", MSc thesis submitted to Cranfield University, UK.

Kapur, K. and Yadav, N. A. (1982), "The effects of certain heavy metal salts on the development of eggs in common carp. Cyprinus carpio var. communis", *Acta Hydrochimica et Hydrobiologica,* vol. 10, no. 5, pp. 517-522.

Kazantzis, G. (2000), "Thallium in the environment and health effects", *Environmental Geochemistry and Health,* vol. 22, pp. 275-280.

Kelly, C.B., (1974), "The Toxicity of Chlorinated Waste Effluents to Fish and Considerations of Alternative Processes for the Disinfection of Waste Effluents," Prepared for the Virginia State Water Control Board, Richmond, Virginia.

Kendall R. L., Anderson T. A., Baker R. J., Bens C. M., Carr J. A., Chiodo L.A., Cobb III G. P., Dickerson R. L., Dixon K. R., Frame L. T., Hooper M. J., Martin C. F., McMurry S. T., Patino R., Smith E. E., Theodorakis C.W. (2001), "Ecotoxicology", In: Casarett and Doull's Toxicology The Basic Science of Poisons. Mc Graw-Hill, Medical Publishing Division, Sixth Edition, pp. 1013-1030

Khaled A.M.M. (2006), "Impact of Heavy metals in aquatic environment", National Institute of oceanography and fisheries, Alexandria (PowerPoint presentation)

Kissa E., Moraitou-Apostolopoulou M. and Kiortsis, V. (1984), "Effects of four heavy metals on survival on hatching rate of Artemia salina (L.) ", *Archiv fur Hydrobiologie,* vol. 102, no.2, pp. 255-264.

Kokkali, V., Katramados I. and Newman, J.D., (2010) "Detecting water toxicity using digital image processing" in *Proceedings of the International Water Association, Young Water Professionals,* UK, pp. 111-117

Koutsaftis A. and Aoyama I. (2007), "Toxicity of four antifouling biocides and their mixtures on the brine shrimp *Artemia salina*", *Science of the Total Environment,* **387**, 166-174.

Kurt, U., Avsar, Y. and Talha Gonullu, M. (2006), "Treatability of water-based paint wastewater with Fenton process in different reactor types", *Chemosphere,* vol. 64, no. 9, pp. 1536-1540.

Lall SP (2002), "The minerals", In: Halver JE, Herdy RW (eds) Fish Nutrition, 3rd edn. Elsevies, Amsterdam, pp 259-308.

Lambrot, R., Livera, G., Coffigny, H., Pairault, C., Frydman, R., Habert, R. and Rouiller-Fabre, V. (2006), "A new method for toxicity assays on human and mouse fetal testis", *Biochimie,* vol. 88, no. 11, pp. 1831-1835.

Lin, S. H. and Chang, C. C. (2000), "Treatment of landfill leachate by combined electro-Fenton oxidation and sequencing batch reactor method", *Water Research,* vol. 34, no. 17, pp. 4243-4249.

Lin, S. H. and Lo, C. C. (1997), "Fenton process for treatment of desizing wastewater", *Water Research,* vol. 31, no. 8, pp. 2050-2056.

Lindsay, J. A. and Holden, M. T. G. (2004), "Staphylococcus aureus: Superbug, super genome?", *Trends in microbiology,* vol. 12, no. 8, pp. 378-385.

Lipczynska-Kochany, E., Sprah, G. and Harms, S. (1995), "Influence of some groundwater and surface waters constituents on the degradation of 4-chlorophenol by the Fenton reaction", *Chemosphere,* vol. 30, no. 1, pp. 9-20.

Liu, J., Goyer R.A., Waalkes M.P. (2001), "Toxic effects of metals", In: Casarett and Doull's Toxicology the Basic Science of Poisons. Mc Graw-Hill, Medical Publishing Division, Sixth Edition, pp.932-959

Lorian, V., (2205), "Antimicrobial susceptibility in solid media", In: Antibiotics in laboratory medicine, Lippincott Williams & Wilkins, USA, Chapter 2, p31

Ługowska K. (2007), "The Effect of cadmium and cadmium/copper mixture during the embryonic development on deformed common carp larvae", *EJPAU*, vol. 10, no. 4, pp. 11.

Lydon, H.L. (2007), "A study of the effect of electrochemical peroxidation treatment on contaminated water samples", MSc thesis submitted to Cranfield University, UK.

Ma, T., Xu, J., Xia, W., Jong, X., Sun, W. and Lin, G. (1992), "Proficiency of the Tradescantia-micronucleus image analysis system for scoring micronucleus frequencies and data analysis", *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis,* vol. 270, no. 1, pp. 39-44.

MacRae T. H. and Pandey A. S. (1991), "Effects of metals on early life stages of the brine shrimp, Artemia: A developmental toxicity assay", *Archives of Environmental Contamination and Toxicology,* vol. 20, no. 2, 247-252.

Moore, D., House, I. and Dixon, A. (1993), "Thallium poisoning", *British medical journal,* vol. 306, no. 6891, pp. 1527-1529.

Murphy, H. (2009), "Measuring the toxicity of wastewater following treatment by electrochemical peroxidation using microbial growth", MSc thesis submitted to Cranfield University, UK.

Murray-Gulde, C. L., Heatley, J. E., Schwartzman, A. L. and Rodgers Jr., J. H. (2002), "Algicidal effectiveness of Clearigate, Cutrine-Plus, and copper sulfate and margins of safety associated with their use", *Archives of Environmental Contamination and Toxicology,* vol. 43, no. 1, pp. 19-27.

Nałęcz-Jawecki, G., Grabińska-Sota, E. and Narkiewicz, P. (2003), "The toxicity of cationic surfactants in four bioassays", *Ecotoxicology and environmental safety,* vol. 54, no. 1, pp. 87-91.

Nealson, K. H. (1977), "Autoinduction of bacterial luciferase. Occurrence, mechanism and significance", *Archives of Microbiology,* vol. 112, no. 1, pp. 73-79.

Newman, J. D., Tigwell, L. J., Warner, P. J. and Turner, A. P. F. (2001), "Biosensors: Boldly going into the new millennium", *Sensor Review,* vol. 21, no. 4, pp. 268-271.

Neyens, E. and Baeyens, J. (2003), "A review of classic Fenton's peroxidation as an advanced oxidation technique", *Journal of Hazardous Materials,* vol. 98, no. 1-3, pp. 33-50.

Nikinmaa M.J. and Schlenk D., (2011), "Aims & Scope", *Aquatic Toxicology*

Noldus, L. P. J. J., Spink, A. J. and Tegelenbosch, R. A. J. (2001), "EthoVision: A versatile video tracking system for automation of behavioral experiments", *Behavior Research Methods, Instruments, and Computers,* vol. 33, no. 3, pp. 398-414.

Nováková J., D. Daňová, K. Strišková, R. Hromada, H. Mičková and M. Rabišková, (2007), "Zinc and Cadmium Toxicity Using a Biotest with *Artemia franciscana*", *Acta Vet. BRNO*, vol. 76, pp. 635.

Nunes B. S., Carvalho F. D., Guilhermino L. M. and Van Stappen G. (2006), "Use of the genus Artemia in ecotoxicity testing", *Environmental Pollution,* vol. 144, pp. 453-462.

Ou Ziqing, Sun Tieheng and Zhang Hairong (1994), "A bioassay for determining simazine in water using aquatic flowering plants (Ceratophyllum oryzetorum, Ranunculus trichophyllus and Alisma plantago-aquatica)", *Pesticide Science,* vol. 42, no. 3, pp. 173-178.

Özcan, A., Şahin, Y., Savaş Koparal, A. and Oturan, M. A. (2008), "Carbon sponge as a new cathode material for the electro-Fenton process: Comparison with carbon felt cathode and application to degradation of synthetic dye basic blue 3 in aqueous medium", *Journal of Electroanalytical Chemistry,* vol. 616, no. 1-2, pp. 71-78.

Palácio, S. M., Espinoza-Quiñones, F. R., Módenes, A. N., Oliveira, C. C., Borba, F. H. and Silva Jr., F. G. (2009), "Toxicity assessment from electro-coagulation treated-textile dye wastewaters by bioassays", *Journal of hazardous materials,* vol. 172, no. 1, pp. 330-337.

Palmqvist, E., Berggren Kriz, C., Khayyami, M., Danielson, B., Larsson, P., Mosbach, K. and Kriz, D. (1994), "Development of a simple detector for microbial metabolism, based on a polypyrrole dc resistometric device", *Biosensors and Bioelectronics,* vol. 9, no. 8, pp. 551-556.

Panagoula, B., Panayiota, M. and Iliopoulou-Georgudaki, J. (2002), "Acute toxicity of TBT and IRGAROL in Artemia salina", *International journal of toxicology,* vol. 21, no. 3, pp.

231-233.

Panizza, M. and Cerisola, G. (2001), "Removal of organic pollutants from industrial wastewater by electrogenerated Fenton's reagent", *Water Research,* vol. 35, no. 16, pp. 3987-3992.

Parsons S. (2004) "Advanced Oxidation Processes for water and Wastewater Treatment", IWA, London pp 111-132.

Parvez, S., Venkataraman, C. and Mukherji, S. (2008), "Toxicity assessment of organic pollutants: Reliability of bioluminescence inhibition assay and univariate QSAR models using freshly prepared Vibrio fischeri", *Toxicology in Vitro,* vol. 22, no. 7, pp. 1806-1813.

Paton, I. K., Lemon, M., Freeman, B. and Newman, J. D. (2009), "Electrochemical peroxidation of contaminated aqueous leachate", *Journal of Applied Electrochemistry, ,* pp. 1-4.

Pelka M., Danzl C., Distler W. and Petschelt A. (2000), "A new screening test for toxicity testing of dental materials", *Journal of dentistry, vol.* 28, no. 5, pp. 341-345.

Piletskaya, E. V., Piletsky, S. A., Sergeyeva, T. A., El'Skaya, A. V., Sozinov, A. A., Marty, J. and Rouillon, R. (1999), "Thylakoid membranes-based test-system for detecting of trace quantities of the photosynthesis-inhibiting herbicides in drinking water", *Analytica Chimica Acta,* vol. 391, no. 1, pp. 1-7.

Polak, M. E., Rawson, D. M. and Haggett, B. G. D. (1996), "Redox mediated biosensors incorporating cultured fish cells for toxicity assessment", *Biosensors and Bioelectronics,* vol. 11, no. 12, pp. 1253-1257.

Portmann R., Leumann M. and Thommen S. (1998), "Method and device for determining toxicity as well as the use thereof", WO95/25955, United States Patent 5,789,242.

Pratap, K. and Lemley, A. T. (1994), "Electrochemical peroxide treatment of aqueous herbicide solutions", *Journal of Agricultural and Food Chemistry,* vol. 42, no. 1, pp. 209-215.

Pratap, K. and Lemley, A. T. (1998), "Fenton Electrochemical Treatment of Aqueous Atrazine and Metolachlor", *Journal of Agricultural and Food Chemistry,* vol. 46, no. 8, pp. 3285-3291.

Prousek, J. (1996), "Advanced oxidation processes for water treatment. Chemical processes", *Chemicke Listy,* vol. 90, no. 4, pp. 229-237.

Quinn, M. (2002), "Electrochemical Peroxidation of Landfill Leachate", MSc Thesis submitted to Cranfield University, UK.

Rand, G. M., Wells, P. G. and McCarty, L.S., (1995), "Introduction to aquatic toxicology", In: Foundamentals of aquatic toxicology. Effects, Environmental fate and risk assessment, (Ed.: Rand, G.M.), Taylor & Francis, Second Edition, Chapter 1, pp. 27-28.

Rawson, D. M., Willmer, A. J. and Turner, A. P. F. (1989), "Whole-cell biosensors for environmental monitoring", *Biosensors,* vol. 4, no. 5, pp. 299-311.

Reemtsma, A.T., Putschew, M. Jekel, (1999), "Industrial wastewater analysis: a toxicity-directed approach", *Waste Manage*m, vol. 19, pp. 181–188.

Renou, S., Givaudan, J. G., Poulain, S., Dirassouyan, F. and Moulin, P. (2008), "Landfill leachate treatment: Review and opportunity", *Journal of Hazardous Materials,* vol. 150, no. 3, pp. 468-493.

Roe, B. A. and Lemley, A. T. (1997), "Treatment of two insecticides in an electrochemical Fenton system", *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes,* vol. 32, no. 2, pp. 261-281.

Ruiz, E. F., Rabago, V. M. E., Lecona, S. U., Perez, A. B. and Ma, T. (1992), "Tradescantia-micronucleus (Trad-MCN) bioassay on clastogenicity of wastewater and in situ monitoring", *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis,* vol. 270, no. 1, pp. 45-51.

Sahul Hameed, A. S. and Balasubramanian, G. (2000), "Antibiotic resistance in bacteria isolated from Artemia nauplii and efficacy of formaldehyde to control bacterial load", *Aquaculture,* vol. 183, no. 3-4, pp. 195-205.

Sarabia R., Del Ramo J., Varo I., Díaz-Mayans J. and Torreblanca A. (2002), "Comparing the acute response to cadmium toxicity of nauplii from different populations of Artemia", *Environmental Toxicology and Chemistry,* vol. 21, no. 2, pp. 437-444.

Sawyer, C.M. (1976), "Wastewater Disinfection: A state-of-the-Art summary", Virginia Water Resources Research Center, Virginia Polytechnic Institute and State University Blacksburg, Virginia 24061 Department of Civil Engineering Virginia VIilitary Institute

Scheerer, S., Gomez, F. and Lloyd, D. (2006), "Bioluminescence of Vibrio fischeri in continuous culture: Optimal conditions for stability and intensity of photoemission", *Journal of Microbiological Methods,* vol. 67, no. 2, pp. 321-329.

Schrock M., James R., Dindal A., Willenberg Z., Riggs K. (2006), "Environmental Technology Verification Report", Battelle, Columbus, Ohio, 43201

Scrudato, R., Chiarenzelli, J. (2000), "Electrochemical peroxidation of contaminated liquids and slurries", WO 00/17113, United States Patent 6045707.

Sikorska, J. and Wolnicki, J. (2010), "Cadmium and copper toxicity to tench Tinca tinca (L.) larvae after a short-term exposure", *Reviews in Fish Biology and Fisheries,* vol. 20, no. 3, pp. 417-423.

Smith, H., (1978), "The molecular biology of plant cells", *University of California*, Los Angeles, USA, chapter 4, pp. 94.

Song, M.Y. and Brown, J.J., (1998), "Osmotic effects as a factor modifying insecticide toxicity on Aedes and Artemia", *Ecotoxicol Environ Saf*., vol 41, no 2, pp. 195-202.

Sorgeloos, P., Remiche van der Wielen, C. and Persoone, G. (1978), "The use of Artemia nauplii for toxicity tests - a critical analysis", *Ecotoxicology and environmental safety,* vol. 2, no. 3-4, pp. 249-255.

Srivastava, R., Kumar, D., Gupta, S.K., 2005. "Bioremediation of municipal sludge by vermitechnology and toxicity assessment by *Allium cepa*", *Bioresour. Technol*., vol. 96, pp. 1867–1871.

Svensson B., Mathiasson L., Mårtensson L. and Bergström S. (2005), "*Artemia salina* as test organism for assessment of acute toxicity of leachate water from landfills", *Environmental monitoring and assessment, vol.* 102, pp. 309-321.

Svobodova Z, Lloyd R, Machova J, Vykusova B (1993), "Water quality and fish health", *EIFAC Tech Pap,* 54, Rome, FAO, p 59.

Tang, W. Z. and Tassos, S. (1997), "Oxidation kinetics and mechanisms of trihalomethanes by Fenton's reagent", *Water Research,* vol. 31, no. 5, pp. 1117-1125.

Tarr, M. A. (2003) **"**Chemical degradation methods for wastes and pollutants: environmental and industrial applications"**,** M. Dekker, New York.

Todar, K. (2008), "Textbook of Bacteriology", Online source www.textbookofbacteriology.net

Tothill, I. E. and Turner, A. P. F. (1996), "Developments in bioassay methods for toxicity testing in water treatment", *TrAC - Trends in Analytical Chemistry,* vol. 15, no. 5, pp. 178-188.

Treece G.D., (2000), "Artemia production for marine larval fish culture", Southern Regional Aquaculture Center (SRAC) Publication, No 702.

United States Environmental Protection Agency (1987), "Ambient water quality criteria for zinc", United States Environmental Protection Agency, Office of Water, Washington, DC

United States Environmental Protection Agency (2001), "Update of ambient water quality criteria for cadmium", United States Environmental Protection Agency, Office of Water, Washington,DC

United States Environmental Protection Agency (2003), "Draft update of ambient water quality criteria for copper", United States Environmental Protection Agency, Washington, DC

Vanhaecke P. and Persoone G. (1984), "The ARC-test: a standardized short-term routine toxicity test with *Artemia* nauplii: methodology and evaluation", In: Ecotoxicological

testing for the marine environment, vol.2. Persoone G., Jaspers E. and Claus C. (Eds). State Univ. Ghent, Belgium; Inst. Mar. Sci. Res., Bredene, Belgium, pp. 143-157.

Vanhaecke, P., Persoone, G., Claus, C. and Sorgeloos, P. (1981), "Proposal for a short-term toxicity test with Artemia nauplii", *Ecotoxicology and environmental safety,* vol. 5, no. 3, pp. 382-387.

Varó I., Navarro J.C. Amat F. and Guilhermino L. (2002a), "Characterisation of cholinesterases and evaluation of the inhibitory potential of chlorpyrifos and dichlorvos to *Artemia salina* and *Artemia parthenogenetica*", *Chemosphere*, vol. 48, 563-569

Varó I., Serrano R., Pitarch E., Amat F., Lopez F.J. and Navarro, J.C. (2002b), "Bioaccumulation of chlorpyrifos through an experimental food chain: study of protein HSP70 as biomarker of sublethal stress in fish", *Archives of Environmental Contamination and Toxicology*, vol. 42, 229-235.

Venkatadri, R. and Peters, R. W. (1993), "Chemical oxidation technologies: Ultraviolet light/hydrogen peroxide, Fenton's reagent, and titanium dioxide-assisted photocatalysis", *Hazardous Waste and Hazardous Materials,* vol. 10, no. 2, pp. 107-149.

Venkateswara Rao J., Kavitha P., Jakka N. M., Sridhar V. and Usman P. K. (2007). "Toxicity of organophosphates on morphology and locomotor behavior in brine shrimp, *Artemia salina*", *Archives of Environmental Contamination and Toxicology,* vol. 53, no. 2, 227-232.

Ventrella, A. (2007), "Photosynthetic membranes and proteins: studies on the factors influencing structural organization and functionality, towards biotechnological applications". PhD thesis submitted to the University of Bari, Italy.

Ventura, A., Jacquet, G., Bermond, A. and Camel, V. (2002), "Electrochemical generation of the Fenton's reagent: Application to atrazine degradation", *Water Research,* vol. 36, no. 14, pp. 3517-3522.

Vigers G.A., Ellis B.M., (1977), "Assessment of the toxicity of landfill leachates by the residual oxygen bioassay", Proc. 3rd Aquatic Toxicity Workshop, Halifax, NS., Nov.2-3, 1976, Environmental Protection Service Technical Report No.EPS-5-AR-77-1, 87-106 .

Waldichuk, M., (1977), "Global Marine Pollution: An Overview", Paris, USESCO 1

Walker P.A., Adrian DD., (1977), "Specific conductance as a measure of treatability of landfill leachates in algal lagoons", Waste Conf., Purdue University, pp. 558-566.

Walling, C. (1975), "Fenton's reagent revisited", *Accounts of Chemical Research,* vol. 8, no. 4, pp. 125-131.

Walling, C. and Goosen, A. (1973), "Mechanism of the ferric ion catalyzed decomposition of hydrogen peroxide. Effect of organic substrates", *Journal of the American Chemical Society,* vol. 95, no. 9, pp. 2987-2991.

Walling, C. and Kato, S. (1971), "The oxidation of alcohols by Fenton's reagent. The effect of copper ion", *Journal of the American Chemical Society,* vol. 93, no. 17, pp. 4275-4281.

Weiling, Z., Lin, Y., Xuancheng, X. and Shuchun, G, (1991), "Toxic effects of zinc on four species of freshwater fish", *Chinese Journal of Oceanology and Limnology,* vol. 9, no. 1, pp. 64-70.

Williams (2008) "Cells- The building Bricks of Life", [Online], accessible at http://www.williamsclass.com/SeventhScienceWork/CellTheoryParts.htm, [Accessed, 5th of January 2009]

Wiszniowski, J., Robert, D., Surmacz-Gorska, J., Miksch, K. and Weber, J. V. (2006), "Landfill leachate treatment methods: A review", *Environmental Chemistry Letters,* vol. 4, no. 1, pp. 51-61.

Yoon, J., Lee, Y. and Kim, S, (2001), "Investigation of the reaction pathway of OH radicals produced by Fenton oxidation in the conditions of wastewater treatment", *Water Science and Technology,* vol. 44, no. 5, pp. 15-21.

Internet sources:

http://bglarochelle.pbworks.com/w/page/5089496/photosynthesis [Accessed, 18th of January 2011]

http://www.bio.miami.edu/dana/226/226F08_10.html [Accessed, 18th of January 2011]

APPENDICES

Appendix A. Statistical Tables

ANOVA tables

Table A-1 Chloroplast assay for atrazine after 5 min of illumination

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	4	2.67020	0.66755	9.66	0.002
Residual	10	0.69116	0.06912		
Total	14	3.36136			

Table A-2 Chloroplast assay for atrazine after 10 min of illumination

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	4	2.823431	0.705858	94.72	<.001
Residual	10	0.074520	0.007452		
Total	14	2.897951			

Table A-3 Chloroplast assay for Cu^{2+} after 5 min of illumination

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	4	0.019272	0.004818	1.66	0.234
Residual	10	0.028989	0.002899		
Total	14	0.048261			

Table A-4 Chloroplast assay for Cu^{2+} after 10 min of illumination

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	4	0.176548	0.044137	43.70	<.001
Residual	10	0.010100	0.001010		
Total	14	0.186648			

Table A-5 Chloroplast assay for phenol after 5 min of illumination

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	4	0.0005711	0.0001428	0.35	0.837
Residual	10	0.0040627	0.0004063		
Total	14	0.0046337			

Table A-6 Chloroplast assay for phenol after 5 min of illumination

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	4	0.0023943	0.0005986	2.29	0.131
Residual	10	0.0026093	0.0002609		
Total	14	0.0050036			

Table A-7 Chloroplast assay for sodium hypochlorite after 5 min of illumination

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	4	0.0052844	0.0013211	2.13	0.152
Residual	10	0.0062080	0.0006208		
Total	14	0.0114924			

Table A-8 Chloroplast assay for sodium hypochlorite after 10 min of illumination

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	4	0.013028	0.003257	0.41	0.801
Residual	10	0.080315	0.008032		
Total	14	0.093343			

Table A-9 Percentage mortality of the nauplii in copper solutions

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	4	24300.31	6075.08	242.12	<.001
Residual	10	250.91	25.09		
Total	14	24551.22			

Table A-10 Percentage mortality of the nauplii in phenol solutions

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	5	24250.00	4850.00	291.00	<.001
Residual	12	200.00	16.67		
Total	17	24450.00			

Table A-11 Percentage mortality of the nauplii in sodium hypochlorite solutions

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	4	31226.67	7806.67	292.75	<.001
Residual	10	266.67	26.67		
Total	14	31493.33			

Table A-12 Percentage inhibition of the mobility of the nauplii monitored by the intial device in atrazine solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	4	1403.7	350.9	0.65	0.640
Residual	10	5400.8	540.1		
Total	14	6804.6			

Table A-13 Percentage inhibition of the mobility of the nauplii monitored by the initial device in copper ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	4	7823.7	1955.9	11.51	<.001
Residual	10	1698.9	169.9		
Total	14	9522.6			

Table A-14 Percentage inhibition of the mobility of the nauplii monitored by the initial device in phenol solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	5	23996.79	4799.36	194.80	<.001
Residual	12	295.64	24.64		
Total	17	24292.44			

Table A-15 Percentage inhibition of the mobility of the nauplii monitored by the initial device in sodium hypochlorite solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	4	9312.46	2328.11	30.01	<.001
Residual	10	775.76	77.58		
Total	14	10088.22			

Table A-16 Percentage inhibition of the mobility of the nauplii monitored by final device in potassium dichromate solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	3	13778.5	4592.8	15.72	0.001
Residual	8	2336.7	292.1		
Total	11	16115.1			

Table A-17 Percentage inhibition of the mobility of the nauplii monitored by final device in various temperatures for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Temperature	3	7292.0	2430.7	5.56	0.003
Time	4	9135.7	2283.9	5.22	0.002
Temperature. Time	12	8549.5	712.5	1.63	0.122
Residual	40	17488.4	437.2		
Total	59	42465.4			

Table A-18 Percentage inhibition of the mobility of the nauplii monitored by final device in potassium dichromate solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	4	31400.5	7850.1	35.85	<.001
Residual	20	4379.7	219.0		
Total	24	35780.2			

Table A-19 Percentage mortality of the nauplii in potassium dichromate solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	4	21350.0	5337.5	10.68	<.001
Residual	20	10000.0	500.0		
Total	24	31350.0			

Table A-20 Percentage inhibition of the mobility of the nauplii monitored by final device in cadmium ion solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	38151.7	5450.2	36.19	<.001
Residual	24	3614.9	150.6		
Total	31	41766.6			

Table A-21 Percentage immortality of the nauplii monitored in cadmium ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	18886.72	2698.10	59.20	<.001
Residual	24	1093.75	45.57		
Total	31	19980.47			

Table A-22 Percentage inhibition of the mobility of the nauplii monitored by final device in copper ion solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	6	19310.0	3218.3	18.68	<.001
Residual	21	3617.8	172.3		
Total	27	22927.8			

Table A-23 Percentage mortality of the nauplii in copper ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	6	32455.4	5409.2	51.93	<.001
Residual	21	2187.5	104.2		
Total	27	34642.9			

Table A-24 Percentage inhibition of the mobility of the nauplii monitored by final device in ferrous ion solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	19541.1	2791.6	23.61	<.001
Residual	24	2838.0	118.2		
Total	31	22379.1			

Table A-25 Percentage inhibition of the mobility of the nauplii monitored by final device in zinc ion solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	13469.5	1924.2	8.94	<.001
Residual	24	5166.1	215.3		
Total	31	18635.6			

Table A-26 Percentage mortality of the nauplii monitored in zinc ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	8417.97	1202.57	12.31	<.001
Residual	24	2343.75	97.66		
Total	31	10761.72			

Table A-27 Percentage inhibition of the mobility of 4 nauplii monitored by final device in aldicarb solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	23835.0	3405.0	22.85	<.001
Residual	16	2384.6	149.0		
Total	23	26219.6			

Table A-28 Percentage inhibition of the mobility of 8 nauplii monitored by final device in aldicarb solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	30593.77	4370.54	88.60	<.001
Residual	16	789.23	49.33		
Total	23	31383.00			

Table A-29 Percentage mortality of 4 nauplii monitored in aldicarb solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	17528.91	2504.13	59.36	<.001
Residual	16	675.00	42.19		
Total	23	18203.91			

Table A-30 Percentage mortality of 8 nauplii monitored in aldicarb solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	17122.85	2446.12	115.96	<.001
Residual	16	337.50	21.09		
Total	23	17460.35			

Table A-31 Percentage mortality of 4 and 8 nauplii in aldicarb solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	33937.21	4848.17	153.23	<.001
Nauplius number	1	23.73	23.73	0.75	<.001
Concentrationx Nauplius number	7	714.55	102.08	3.23	<.001
Residual	32	1012.50	31.64		
Total	47	35687.99			

Table A-32 Percentage inhibition of the mobility of 4 and 8 nauplii in aldicarb solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	53266.01	7609.43	76.72	<.001
Nauplius number	1	3485.94	3485.94	35.15	<.001
Concentrationx Nauplius number	7	1162.73	166.10	1.67	0.151
Residual	32	3173.86	99.18		
Total	47	61088.53			

Table A-33 Percentage mortality of 4 nauplii in colchicine solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	4384.50	626.36	29.69	<.001
Residual	16	337.51	21.09		
Total	23	4722.01			

Table A-34 Percentage mortality of 8 nauplii in colchicine solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	4282.03	611.72	12.89	<.001
Residual	16	759.38	47.46		
Total	23	5041.41			

Table A-35 Percentage mortality of 4 and 8 nauplii in colchicine solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	7705.92	1100.85	32.12	<.001
Nauplius number	1	1277.20	1277.20	37.26	<.001
Concentrationx Nauplius number	7	960.61	137.23	4	0.003
Residual	32	1096.88	34.28		
Total	47	11040.62			

Table A-36 Percentage inhibition of the mobility of 4 nauplii monitored by final device in colchicine solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	30545.8	4363.7	34.19	<.001
Residual	16	2042.3	127.6		
Total	23	32588.1			

Table A-37 Percentage inhibition of the mobility of 8 nauplii monitored by final device in colchicine solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	35452.61	5064.66	165.52	<.001
Residual	16	489.57	30.60		
Total	23	35942.18			

Table A-38 Percentage inhibition of the mobility of 4 and 8 nauplii in colchicine solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	65153.94	9307.71	117.64	<.001
Nauplius number	1	146.91	146.91	1.86	0.183
Concentrationx Nauplius number	7	844.49	120.64	1.52	0.194
Residual	32	2531.83	79.12		
Total	47	68677.17			

Table A-39 Percentage inhibition of the mobility of 4 nauplii monitored by final device in thallium sulphate solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	28681.3	4097.3	23.28	<.001
Residual	16	2816.0	176.0		
Total	23	31497.3			

Table A-40 Percentage inhibition of the mobility of 8 nauplii monitored by final device in thallium sulphate solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	36732.5	5247.5	35.77	<.001
Residual	16	2347.3	146.7		
Total	23	39079.8			

Table A-41 Percentage inhibition of the mobility and of 4 and 8 nauplii in thallium sulphate solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	63363.0	9051.9	56.10	<.001
Nauplius number	1	972.6	972.6	6.03	0.020
Concentrationx Nauplius number	7	2050.8	293.0	1.82	0.118
Residual	32	5163.3	161.4		
Total	47	71549.7			

Table A-42 Percentage mortality of 4 nauplii in thallium sulphate solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	32210.16	4601.45	109.07	<.001
Residual	16	675.00	42.19		
Total	23	32885.16			

Table A-43 Percntage mortality of 8 nauplii in thallium sulphate solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	13030.66	1861.52	117.67	<.001
Residual	16	253.12	15.82		
Total	23	13283.79			

Table A-44 Percentage mortality of the mobility of 4 and 8 nauplii in thallium sulphate solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	39906.74	5700.96	196.56	<.001
Nauplius number	1	1647.95	1647.95	56.82	<.001
Concentrationx Nauplius number	7	5334.08	762.01	26.27	<.001
Residual	32	928.12	29.00		
Total	47	47816.89			

Table A-45 Percentage inhibition of the mobility of 4 nauplii monitored by final device in paraoxon-ethyl solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	45154.74	6450.68	160.89	<.001
Residual	16	641.51	40.09		
Total	23	45796.25			

Table A-46 Percentage inhibition of the mobility of 8 nauplii monitored by final device in paraoxon-ethyl solutions for video duration of 2 min

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	45206.12	6458.02	121.21	<.001
Residual	16	852.46	53.28		
Total	23	46058.59			

Table A-47 Percentage inhibition of the mobility of 4 and 8 nauplii in paraoxon-ethyl solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	90059.08	12865.58	275.57	<.001
Nauplius number	1	350.27	350.27	7.50	0.010
Concentrationx Nauplius number	7	301.79	43.11	0.92	0.502
Residual	32	1493.98	46.69		
Total	47	92205.11			

Table A-48 Percentage mortality of 4 nauplii in paraoxon-ethyl solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	30599.0	4371.3	41.96	<.001
Residual	16	1666.7	104.2		
Total	23	32265.6			

Table A-49 Percentage mortality of 8 nauplii in paraoxon-ethyl solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	25307.23	3615.32	45.70	<.001
Residual	16	1265.62	79.10		
Total	23	26572.85			

Table A-50 Percentage mortality of 4 and 8 nauplii in paraoxon-ethyl solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	54917.94	7845.42	85.62	<.001
Nauplius number	1	541.70	541.70	5.91	0.021
Concentrationx Nauplius number	7	988.25	141.18	1.54	0.189
Residual	32	2932.29	91.63		
Total	47	59380.18			

Table A-51 Percentage inhibition of the mobility of 4 and 8 nauplii in cadmium ion solutions after 16,20 and 24 hours of incubation

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	123378.1	17625.4	119.05	<.001
Nauplius number	1	64.4	64.4	0.43	0.511
Time	2	129.4	64.7	0.44	0.647
Concentration x Nauplius number	7	3376.9	482.4	3.26	0.003
Concentration. time	14	3808.7	272.0	1.84	0.038
Nauplius numberx Time	2	114.0	57.0	0.38	0.681
Concentration x Nauplius number x Time	14	2705.8	193.3	1.31	0.211
Residual	144	21319.9	148.1		
Total	191	154897.1			

Table A-52 Percentage inhibition of the mobility of 4 and 8 nauplii in copper ion solutions after 16, 20 and 24 hours of incubation

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	222166.4	31738.1	120.12	<.001
Nauplius number	1	5519.9	5519.9	20.89	<.001
Time	2	6969.5	3484.8	13.19	<.001
Concentration x Nauplius number	7	8096.8	1156.7	4.38	<.001
Concentration. time	14	3740.4	267.2	1.01	0.445
Nauplius numberx Time	2	5054.1	2527.1	9.56	<.001
Concentration x Nauplius number x Time	14	4510.0	322.1	1.22	0.267
Residual	144	38046.7	264.2		
Total	191	294104.0			

Table A-53 Percentage inhibition of the mobility of 4 and 8 nauplii in ferrous ion solutions after 16, 20 and 24 hours of incubation

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	7	213083.4	30440.5	209.11	<.001
Nauplius number	1	389.9	194.9	1.34	0.265
Time	2	1.2	1.2	0.01	0.929
Concentration x Nauplius number	7	2227.7	159.1	1.09	0.369
Concentration. time	14	1954.5	279.2	1.92	0.071
Nauplius numberx Time	2	586.1	293.1	2.01	0.137
Concentration x Nauplius number x Time	14	651.2	46.5	0.32	0.991
Residual	144	20962.0	145.6		
Total	191	239856.0			

Table A-54 Percentage inhibition of the mobility of 4 and 8 nauplii in zinc ion solutions after 16, 20 and 24 hours of incubation

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	7	143673.9	20524.8	130.74	<.001
Nauplius number	1	720.8	720.8	4.59	0.034
Time	2	1138.7	569.3	3.63	0.029
Concentration x Nauplius number	7	3016.5	430.9	2.74	0.011
Concentration. time	14	4074.4	291.0	1.85	0.036
Nauplius numberx Time	2	1260.8	630.4	4.02	0.020
Concentration x Nauplius number x Time	14	2475.4	176.8	1.13	0.340
Residual	144	22606.7	157.0		
Total	191	178967.0			

Table A-55 Percentage inhibition of the mobility of 4 and 8 nauplii in phenol solutions after 16, 20 and 24 hours of incubation

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	7	205076.5	29296.6	169.77	<.001
Nauplius number	1	41.6	41.6	0.24	0.624
Time	2	45.7	22.8	0.13	0.876
Concentration x Nauplius number	7	2069.6	295.7	1.71	0.110
Concentration. time	14	1187.3	84.8	0.49	0.935
Nauplius numberx Time	2	6.1	3.1	0.02	0.982
Concentration x Nauplius number x Time	14	1242.5	88.8	0.51	0.922
Residual	144	24849.2	172.6		
Total	191	234518.4			

Table A-56 Percentage inhibition of the mobility of 4 and 8 nauplii in sodium hypochlorite solutions after 16, 20 and 24 hours of incubation

Source of variation	d.f.	s.s.	m.s.	v.r	F pr
Concentrations	4	171787.70	42946.93	3181.32	<.001
Nauplius number	1	9.02	9.02	0.67	0.416
Time	2	18.82	9.41	0.70	0.501
Concentration x Nauplius number	4	142.83	35.71	2.65	0.039
Concentration. time	8	116.39	14.55	1.08	0.386
Nauplius numberx Time	2	13.83	6.92	0.51	0.601
Concentration x Nauplius number x Time	8	17.61	2.20	0.16	0.995
Residual	90	1214.98	13.50		
Total	119	173321.19			

Table A-57 Percentage inhibition of the mobility of swarm of nauplii monitored by final device in cadmium ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	8	29348.59	3668.57	134.20	<.001
Residual	27	738.11	27.34		
Total	35	30086.69			

Table A-58 Percentage inhibition of the mobility of swarm of nauplii monitored by final device in ferrous ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	8	30829.09	3853.64	53.43	<.001
Residual	27	1947.30	72.12		
Total	35	32776.40			

Table A-59 Percentage inhibition of the mobility of swarm of nauplii monitored by final device in zinc ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	8	13956.54	1744.57	28.01	<.001
Residual	27	1681.41	62.27		
Total	35	15637.96			

Table A-60 Percentage inhibition of the mobility of 4 nauplii of nauplii monitored by final device in ferrous ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	8	31256.5	3907.1	8.69	<.001
Residual	36	16192.5	449.8		
Total	44	47449.0			

Table A-61 Percentage inhibition of the mobility of swarm of nauplii of nauplii monitored by final device in ferrous ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	8	31178.83	3897.35	117.69	<.001
Residual	36	1192.13	33.11		
Total	44	32370.96			

Table A-62 Percentage inhibition of the mobility of a swarm of nauplii and 4 nauplii of nauplii monitored by final device in ferrous ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	8	57239.9	7155.0	29.63	<.001
Nauplius number	1	8128.2	8128.2	33.66	<.001
Concentration x Nauplius number	8	5195.5	649.4	2.69	0.012
Residual	72	17384.6	241.5		
Total	89	87948.1			

Table A-63 Percentage inhibition of the mobility of swarm of nauplii of nauplii monitored by final device in cadmium ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	9	44425.39	4936.15	71.92	<.001
Residual	30	2058.98	68.63		
Total	39	46484.37			

Table A-64 Percentage inhibition of the mobility of swarm of nauplii of nauplii monitored by final device in copper ion solutions

Source of variation	d.f.	s.s.	m.s.	v.r	.F pr
Concentrations	9	37940.28	4215.59	69.32	<.001
Residual	30	1824.42	60.81		
Total	39	39764.70			

Appendix B. Device Manuals

ToxAlert® manual:

ToxAlert®10 Toxicity Test Kit

(Operating Range 10°C to 30°C)

Handy Hints

✓ The Reconstitution Medium and Control Solution take time to thaw. Ensure that the Reconstitution Solution and Control Solution have time to thaw completely before starting the assay.

✓ After thawing, the Reconstitution Medium and Control Solution must be shaken well to completely dissolve all components and to guarantee sufficient oxygen for bioluminescence.

✓ For use in the field smaller volumes of the Reconstitution Medium and Control Solution may be removed and the bottles returned to -20°C.

✓ Add NaCl to the sample at a concentration of 2g per 100ml of sample.

✓ Assay components, the control and the sample must be at the same temperature before starting the assay.

✓ If dilution of the sample is necessary, use 2% NaCl (Control Solution) as the diluent

✓ Do NOT recycle Measurement Cuvettes due to the possibility of cross contamination.

✓ Measurements must be made with caps and rubber bungs removed from all cuvettes.

✓ After use return the Reconstitution Medium and Control Solution to -20°C.

✓ Always store the Reagent long term at -20°C.

Points to Note

✓ Check that the pH of the sample is between 6.0 and 8.0.

✓ To obtain the most comparable results, it is recommended that the assays be performed under similar temperature conditions, eg air conditioned laboratory.

✓ New glass or polypropylene containers must be used for samples and dilutions, and for the Reconstitution Medium and Control Solution when removed from the bottles for field use.

✓ If the sample is very turbid it should be filtered.

✓ Highly saline samples, eg sea water, should be measured against an appropriate artificial sea water control.

✓ For field operation ensure that there is sufficient NaCl in the storage bottle provided.

1. Sample Preparation

The sample is prepared in the same manner, irrespective of the chosen test procedure ie SIN, MULT, EC.

a. In the laboratory

  i. Weigh out the equivalent of 2g NaCl per 100ml of sample.
  ii. Add the required volume of sample to the salt.
  iii. Mix well to ensure that the salt crystals have dissolved.

b. In the field

  i. Carefully add a level measuring spoon of NaCl to an empty Measurement Cuvette.
  ii. Using a pipette, add 2.5ml of sample onto the salt.
  iii. Mix well by aspiration to ensure that the salt crystals have completely dissolved.

c. For sample dilution

  i. Make the appropriate dilutions using 2% NaCl (Control Solution) as the diluent.

2. Reagent Preparation

The reagent is reconstituted in the same manner, irrespective of the chosen test procedure ie. SIN, MULT, EC.

1. Thaw a bottle of Reconstitution Medium and Control Solution (2% NaCl) and allow to equilibrate to ambient temperature. At the same time remove the required number of measurement cuvettes, containing the freeze dried reagent, and allow to equilibrate to ambient temperature.
2. Place the measurement cuvette(s) containing the reagent in row A of the rack. Shake the bottle of Reconstitution Medium well and add 1ml of Reconstitution Medium to each measurement cuvette of reagent. Wait 20 minutes to allow full reconstitution.

3. Test Procedure

a. SIN Assay (SINgle shot Mode)

1. Prepare sample as described in section 1.
2. Prepare reagent as described in section 2.
3. Place an empty measurement cuvette in row C of the rack. Position the cuvette such that it accompanies the cuvette in row A.
4. Whilst the reagent is reconstituting, turn on the luminometer and select SIN mode.
5. If the results are to be stored press STR, select the appropriate site name in which the values are to be stored and press OK. If the results are to be measured and not stored select MEAS.
6. When the reconstitution period of 20 minutes has elapsed, mix the contents of the measurement cuvette in row A by aspiration several times, and transfer 0.5ml reagent to the accompanying vial in row C.
7. Shake the Control Solution well and add 0.5ml to the measurement cuvette in row A and press OK on the luminometer. A count up timer will appear.
8. After 30 seconds add 0.5ml sample solution to the accompanying cuvette in row C, and discard the pipette tip.

Allow to incubate

9. Place the measurement cuvette containing the control solution in row A into the luminometer, and at 15 minutes press LOAD. The instrument will eject the cuvette when the measurement is complete. Return the cuvette to the original position in the rack.
10. Place the cuvette containing the sample, in row C, into the luminometer and at 15 minutes 30 seconds press LOAD. The instrument will automatically eject the cuvette when the measurement is complete. Return the cuvette to the original position in the rack.
11. The % inhibition will be displayed on the screen. If you wish to continue taking measurements press CONT. If you wish to stop the assay press ESC. The timer will continue to run.
12. If CONT was selected the luminometer will prompt you to LOAD the measurement cuvette containing the control solution, and the above procedure is repeated.

b. MULT Assay (MULTiple single shot Mode)

1. Prepare sample as described in section 1.
2. Prepare reagent as described in section 2.
3. Place the required number of empty measurement cuvettes in row C. Position them such that each measurement cuvette in row A is accompanied by an empty cuvette in row C, (ie. in pairs).
4. Whilst the reagent is reconstituting, turn on the luminometer and select MULT mode
5. If the results are to be stored press STR, if the results are to be measured and not stored select MEAS.
6. Set the number of SIN assays to be carried out.
7. If STR was chosen select the appropriate site name(s) in which the data for each sample is to be stored.
8. Set the time delay between the measurement of each cuvette. 30 seconds is recommended. The method described here will follow 30 seconds.
9. When the reconstitution period of 20 minutes has elapsed, mix the contents of the first measurement cuvette in row A by aspiration several times, and transfer 0.5ml reagent to the accompanying vial in row C. Repeat this procedure for each of the measurement cuvettes.
10. Shake the Control Solution well and add 0.5ml to the first measurement cuvette (control 1) in row A and press OK on the luminometer. A count up timer will appear.
11. After 30 seconds add 0.5ml sample solution to the accompanying cuvette in row C and discard the pipette tip.
12. At 1 minute add 0.5ml Control Solution to the second vial in row A (control 2) and at 1 minute 30 seconds add 0.5 ml sample to the accompanying cuvette in row C (sample 2). Continue with this procedure for the remaining cuvettes, maintaining the chosen time interval. NOTE Remember to discard the pipette tip after the addition of each sample.

Allow to incubate

13. Place the measurement cuvette containing the control solution (control 1) in row A into the luminometer, and at 15 minutes press LOAD. The instrument will eject the cuvette when the measurement is complete. Return the cuvette to the original position in the rack
14. Place the cuvette containing the sample of the first pair (sample 1) in row C into the luminometer, and at 15 minutes 30 seconds press LOAD
15. The % inhibition will be displayed on the screen. If you wish to continue taking measurements for this sample press CONT. If you wish to stop taking measurements for this sample press escape
16. Return the sample cuvette to the original position in the rack, and repeat the procedure with all pairs, maintaining the 30 second time interval
17. The procedure is repeated for those samples selected for continuation. The timer continues to run. The luminometer will prompt you to LOAD the next appropriate control cuvette. NOTE Remember to maintain the time interval, making allowance for any samples which have been discontinued.
18. On completion of all tests, ie. ESC pressed after each sample has been read, you will be given the option to view the data. Use the buttons marked with the up and down arrows to scroll and view the results.

d. EC Assay (Effective Concentration Mode)

1. Prepare sample as described in section 1.
2. Prepare reagent as described in section 2.
3. Place the required number of empty measurement cuvettes in the rack, ie one cuvette for the control and one cuvette for each dilution to be tested.
4. Whilst the reagent is reconstituting, turn on the luminometer and select EC mode
5. If the results are to be stored press STR, and select the appropriate site name in which data is to be stored. If the results are to be measured and not stored select MEAS.
6. Set the number of dilutions to be tested. NOTE The undiluted sample, if to be tested, counts as a dilution.
7. When the reconstitution period of 20 minutes has elapsed, pool the reagent from each of the measurement cuvettes in a new glass or polypropylene container and discard the cuvettes.
8. Mix by aspiration.
9. Dispense 0.5ml of the bacterial test suspension into each empty measurement cuvette.
10. Shake the Control Solution well.
11. Place the first measurement cuvette (the control) into luminometer and press LOAD. The light at T_0 is measured and the count up timer starts.
12. The instrument will automatically eject the cuvette when the measurement is complete. Immediately add 0.5ml of Control Solution to the cuvette and return it to the original position in the rack.
13. Place the measurement cuvette for dilution 1 into the luminometer and at 30 seconds press LOAD. The light at T_0 is measured.
14. The instrument will automatically eject the cuvette when the measurement is complete. Immediately add 0.5ml of dilution 1 solution to the cuvette and return it to the original position in the rack. NOTE This must be the most diluted sample. There is no need to discard the pipette tip providing the samples are added in order of increasing concentration, ie. starting with the most dilute.
15. The instrument will automatically eject the cuvette when the measurement is complete. Place the measurement cuvette for dilution 2 into the luminometer and at 1 minute press LOAD. The light at T_0 is measured.
16. Immediately add 0.5ml of dilution 2 solution to the cuvette and return it to the original position in the rack. NOTE This must be the second lowest dilution sample.
17. Continue with this procedure for each of the dilutions in turn, maintaining the 30 second time interval.

Allow to incubate

18. Place the measurement cuvette containing the control solution into the luminometer and at 15 minutes press LOAD. The instrument will eject the cuvette when the measurement is complete. Return the cuvette to the original position in the rack.
19. Place the cuvette containing dilution 1 into the luminometer and at 15 minutes 30 seconds press LOAD. The instrument will automatically eject the cuvette when the measurement is complete. Return the cuvette to the original position in the rack.
20. Place the cuvette containing dilution 2 into the luminometer and at 16 minutes press LOAD. The instrument will automatically eject the cuvette when the measurement is complete.
21. Continue this procedure for each of the dilutions in turn, maintaining the 30 second time interval.
22. After the measurement of the last dilution the % inhibition is calculated for each dilution. The % Inhibition for the first dilution is displayed on the screen. Using the buttons denoted with the up and down arrows you may scroll and view the results.
23. The timer continues to count up. When you have finished viewing the results press OK.
24. If you wish to continue taking measurements press CONT and the procedure is repeated. If you wish to stop taking measurements press ESC.

Appendix C. Publications

Article

Monitoring the Effect of Metal Ions on the Mobility of *Artemia salina* Nauplii

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Abstract: This study aims to measure the effect of toxic aqueous solutions of metals on the mobility of *Artemia salina* nauplii by using digital image processing. The instrument consists of a camera with a macro lens, a dark chamber, a light source and a laptop computer. Four nauplii were inserted into a macro cuvette, which contained copper, cadmium, iron and zinc ions at various concentrations. The nauplii were then filmed inside the dark chamber for two minutes and the video sequence was processed by a motion tracking algorithm that estimated their mobility. The results obtained by this system were compared to the mortality assay of the *Artemia salina* nauplii. Despite the small number of tested organisms, this system demonstrates great sensitivity in quantifying the mobility of the nauplii, which leads to significantly lower EC_{50} values than those of the mortality assay. Furthermore, concentrations of parts per trillion of toxic compounds could be detected for some of the metals. The main novelty of this instrument relies in the sub-pixel accuracy of the tracking algorithm that enables

robust measurement of the deterioration of the mobility of *Artemia salina* even at very low concentrations of toxic metals.

1. Introduction

The ongoing interest in assessing the environmental effect of toxic waste materials has led to an increasing number of bioassays and screening devices. Such techniques are often based on measuring the effect of toxic substances on the behaviour and life cycle of microorganisms, invertebrates and algae. Different biological properties that have been utilized for toxicity screening include the luminescence of *Vibrio fischeri*, respiratory rate of aerobic bacteria, enzyme activity of *Dapnia magna*, fluorescence of *Chlorella vulgari (algae)* and the lethality to crustaceans (such as *Artemia salina*).

In addition, several studies have specifically focused on the impact of metals to the biota, by analysing their toxic effect to crustaceans. Giarratano *et al*. [1] and Lorenzon *et al*. [2] have investigated the effect of heavy metals to *Exosphaeroma gigas* and *Palaemon elegans* respectively. Furthermore, MacRae and Pandey [3] have researched the relation between water toxicity and hatching success of *Artemia* species. Similarly, Gajbhiye and Hirota [4] have proved that the lethality of these species is dependent on the concentration of heavy metals in water.

Artemia species, or brine shrimps, have also been used in many scientific experiments for acute toxicity testing of toxic materials including pesticides (Barahona & Sánchez-Fortún [5]), leachates (Svensson *et al*. [6]), dental materials (Pelka *et al*. [7]), fungal toxins (Harwig & Scott, [8]) and antifouling biocides (Koutsaftis and Aoyama [9]). Although *Artemia* species are not considered as sensitive as other screening instruments or organisms (Nunes *et al*. [10]), they have some important advantages including constant commercial availability all year round, cost-efficient, easy to culture, short life-cycle, no feeding required during the assay and great offspring production (Vanhaecke *et al*. [11]).

These advantages have led to a wide range of *Artemia*-based bioassays. The determination of the LC_{50} of the nauplii (instar II-III stage) (Vanhaecke and Persoone [12]), the hatchability of the cysts (MacRae and Pandey [3]), the different age specimens (Barahona and Sánchez-Fortún, [5]) and the disruptions on an enzyme property (Varó *et al*. [13,14]) are only some of the *Artemia* end-points that have already been examined as evidence of toxicity. Specifically, Vanhaecke and Persoone [12] have demonstrated a shrimp-based bioassay, known as the ARC-test (*Artemia* Reference Centre), with highly reproducible and accurate results. Additionally, Vanhaecke *et al*. [15] proved that the most sensitive age for the majority of the tested compounds were the 48-h old nauplii at the stage of instar II-III. The same results have also been supported by Barahona and Sánchez-Fortún [5] and Togulga [16]. On the other hand, *Artemia* species are very resistant to metals and accumulate them without any obvious effect on their life-cycle (Sarabia *et al*. [13]).

This study is based on a patented device by Portmann *et al*. [17], which has been redesigned to be more accurate in recording small changes in the mobility of *Artemia salina* nauplii using a monocular camera and a digital image-processing algorithm. The main hypothesis of this work was that the accumulation of toxic compounds to *Artemia* organism has a deteriorating effect on its mobility and this deterioration can be measured. Potassium dichromate was firstly tested as a reference substance since it is a widely applied reference toxic compound in aquatic toxicology (Vanhaecke *et al*. [15]). Later, copper, cadmium, iron and zinc salts were tested for their lethal effect on *Artemia salina* nauplii. Furthermore, the survivors were also counted and their hypothetical mobility was estimated. Finally, the mobility of the nauplii in those toxic metal samples was recorded and compared to the hypothetical one. The results showed that concentrations of parts per trillion of

toxic metals affected the mobility of the nauplii and these changes could be detected consistently using the proposed technique.

2. Materials and Methods

2.1. Preparation of the Test Organisms

Artemia cysts (San Francisco Strain Brine shrimps) were incubated in artificial seawater (ASW) of 3.5% salinity, at 30 °C, pH 8.0 ± 0.5 and under constant aeration for 48 h until they reach the stage of instar II-III. The temperature of the samples throughout the experiment remained at 25 °C. Nauplii from the same generation was applied for each compound.

2.2. Test Chemicals

The reagents were of general laboratory grade unless stated otherwise. Potassium dichromate was provided from BDH (Lutterworth, UK). Copper sulphate [$CuSO_4 \cdot 5H_2O$] and cadmium chloride [$CdCl_2 \cdot H_2O$] were obtained from Sigma-Aldrich (Gillingham, UK). Copper nitrate [$Cu (NO_3)_2 \cdot 3H_2O$] was provided from Fisher Scientific (Loughborough, UK). Zinc sulphate [$ZnSO_4 \cdot 7H_2O$] and iron sulphate [$FeSO_4 \cdot 7H_2O$] were obtained from Acros Organics (Loughborough, UK). Stock solutions of 10,000 mg/L of all reagents were prepared in distilled water and then serial dilutions in ASW were prepared for each metal. The pH of the samples was 8.0 ± 0.5. Macro-cuvettes from polystyrene were used as containers for the toxic samples provided by Fischer Scientific. The same samples of each toxic compound were applied for the assessment of the toxicity using the mortality based assay, hypothetical mobility and mobility assay.

2.3. Further Equipment

A monocular camera (Canon Power shot S3 IS) and a laptop were used to track the movement of the nauplii by recording their activity for two minutes and processing the videos. Light-emitting diodes (LED) torches were used as illumination source. Subsequently, the captured video sequence was analysed by a digital image processing algorithm that estimates the mobility of the nauplii. The concept has been based on the U.S. Patent of Portmann *et al.* [17].

2.4. Mortality-Based Assay

The *Artemia* cysts were cultured in ASW until the stages of instar II-III nauplii (48 h old). Four mL of either ASW (controls) or the tested compound was added to each cell. Then, four nauplii were transferred with Pasteur pipette with the minimal of ASW carried over. Five replicates were performed for the controls and four for every toxicant dilution. After 24 h of incubation, the dead nauplii were counted and the LC_{50} values estimated with probit analysis. The assay would be considered valid if the mortality percentage of the control does not exceed the 10% (Vanhaecke *et al.* [11]) and the
estimated LC_{50} of the reference substance (potassium dichromate) ranges between 30 and 50 mg/L (Svensson *et al.* [6]).

2.5. Hypothetical Mobility of the Nauplii

The main advantage of the proposed approach is that it can quantitatively measure subtle changes in the mobility of nauplii and as a result detect small concentrations of toxic substances. Two
minute-videos of five replicates of four nauplii in ASW were recorded and processed through the digital image processing algorithm. In order to calculate the "% hypothetical mobility" of a specific sample, the following equation was applied (Equation (1)).

$$\% \ Hypothetical \ mobility = \frac{N_{24}}{N_0} \times 100 \tag{1}$$

where N_{24} is the number of the nauplii which remained alive after 24 h of exposure to the toxic substance and N_0 is the average number of the nauplii initially inserted into the control sample. This approach essentially makes the assumption that alive nauplii have retained 100% of their initial mobility after 24 h in the toxic solutions. In Figure 1, the hypothetical mobility is plotted against the actual mobility of the nauplii. The corresponding "best fit model" equations are displayed on Table 1.

Figure 1. Percentage of (o) hypothetical and (●) measured mobility (using the camera-based instrument) of the *Artemia salina* nauplii for Cadmium, Iron, Copper and Zinc ions. The error bars represent the standard error from the four replicates of each concentration.

Cd^{2+}

Cu^{2+}

Fe^{2+}

Zn^{2+}
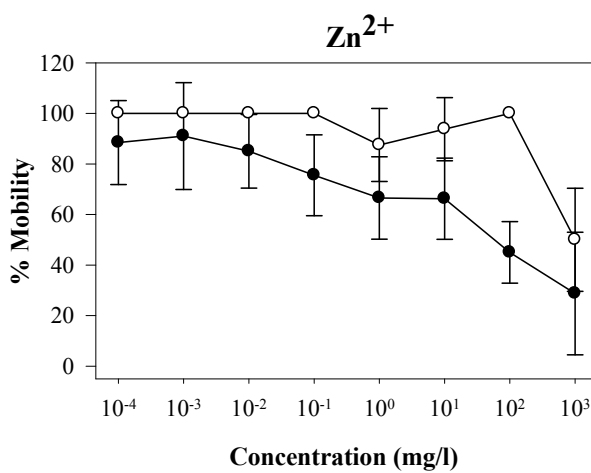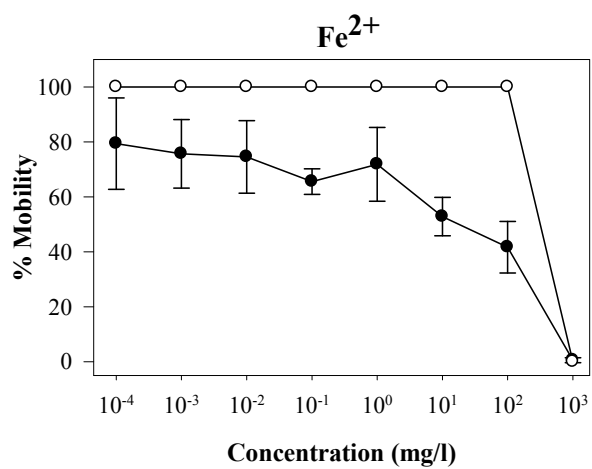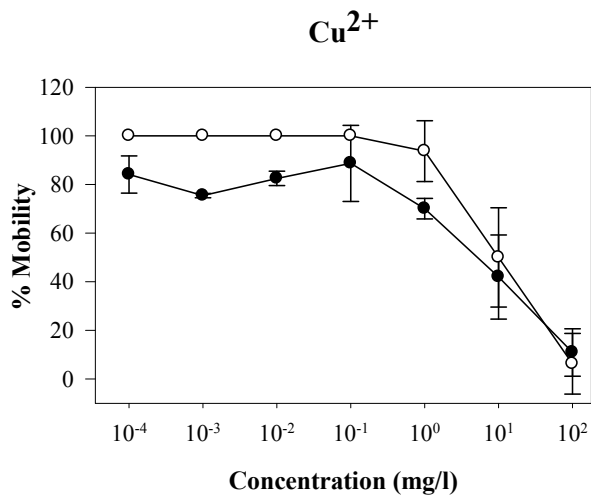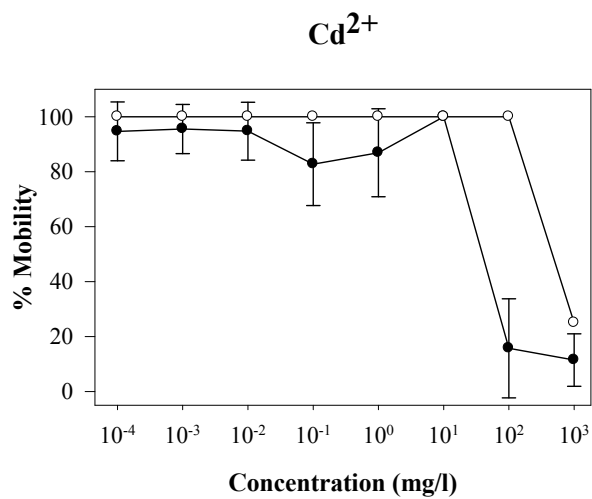
Table 1. Equations from the curves of hypothetical and measured mobility using the "best fit model".

Compound	Equation and R^2 from %mobility graphs	Equation and R^2 from %hypothetical mobility graphs
Cadmium chloride	$y = 0.0008x^2 - 0.8406x + 93.713$ $R^2 = 0.9606$	$y = -0.00008x^2 + 0.0084x + 99.987$ $R^2 = 1$
Copper sulphate	$y = 0.0366x^2 - 4.3644x + 81.278$ $R^2 = 0.9683$	$y = 0.0452x^2 - 5.4572x + 99.97$ $R^2 = 0.9999$
Iron sulphate	$y = 0.0003x^2 - 0.3341x + 70.83$ $R^2 = 0.9267$	$y = -0.0001x^2 + 0.0112x + 99.983$ $R^2 = 1$
Zinc sulphate	$y = 0.0003x^2 - 0.3933x + 79.705$ $R^2 = 0.8493$	$y = -0.00008x^2 + 0.0344x + 96.893$ $R^2 = 0.9336$

*NE: Not Estimated.

2.6. Mobility Assay Using Digital Image Processing

Video sequences were recorded for two minutes for all the controls and samples. All the cells were properly illuminated with LED torches (AAA battery torch, 7dayshop, Guernsey). In each video sequence, the nauplii were counted and tracked ass illustrated in Figure 2. The % mobility of the nauplii in each sample was estimated according to the following Equation (2):

$$\% \, Mobility \; = \frac{\bar{V}_{24}}{\bar{V}_0} \times 100 \tag{2}$$

where $\bar{V}_{24}$ is the average speed per nauplius in a specific sample after 24 h of exposure to the toxicant as was estimated automatically from the algorithm and $\bar{V}_0$ is the average speed per nauplius in the control samples.

Figure 2. The left image shows a representative video frame as captured by the camera. A sequence of such frames forms the input to the digital image processing algorithm. Although the shrimps are not clearly visible in the cuvette, they can be tracked with good accuracy in order to measure their mobility. The remaining images show some characteristic examples of tracking paths which were derived over 6.6 s intervals. Lighter gray shades denote areas of higher mobility including overlapping tracking paths
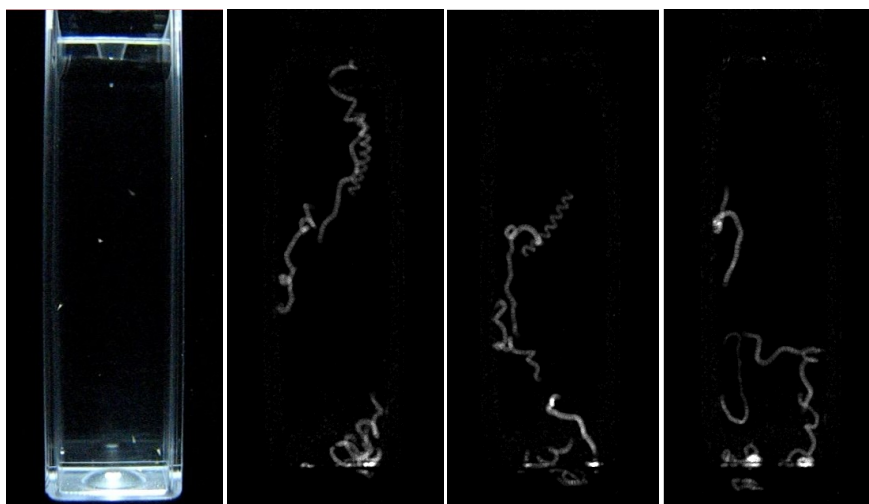
The Effective Concentration (EC_{50}) was estimated through the "best fit model" (Excel, Microsoft Office, 2007). The best fit model revealed that the most appropriate equation was the quadratic equation (Table 1).

2.7. Statistical Analysis

Statistical analysis of one-way ANOVA of the data was performed using Genstat v.12.0 (VSN International Ltd, Hemel Hempstead, UK) and was evaluated at the $p < 0.001$ level, as presented in Table 2. Furthermore, the 24 h LC_{50} was determined by using Biostat v.5.2.5.0 (AnalystSoft, 2008). Finally, two-sample t-test was performed for the results obtained from the hypothetical and actual mobility of the nauplii and they were evaluated for their significance at the $p < 0.05$ level using SigmaPlot v.11.0 (Systat Software, 2008)

Table 2. One-way ANOVA table of the mobility of the nauplii monitored in Cadmium, Iron, Copper and Zinc solutions.

Substance	Source of variation	Degrees of freedom	Sums of squares	Mean square	Variance ratio	F probability
Cadmium	Concentrations	7	38,151.7	5,450.2	36.19	<0.001
	Residual	24	3,614.9	150.6		
	Total	31	41,766.6			
Copper	Concentrations	6	19,310.0	3,218.3	18.68	<0.001
	Residual	21	3,617.8	172.3		
	Total	27	22,927.8			
Iron	Concentrations	7	19,541.1	2,791.6	23.61	<0.001
	Residual	24	2,838.0	118.2		
	Total	31	22,379.1			

Zinc	Concentrations	7	13,469.5	1,924.2	8.94	<0.001
	Residual	24	5,166.1	215.3		
	Total	31	18,635.6			

3. Results and Discussion

The results from the mortality assay of *Artemia salina* nauplii at the stage of instar II-III were considered valid since the mortality percentage of the controls was 0%. The first experiment regarding the mobility assay was to record samples with ASW and 8 nauplii at $time_0$ and $time_{24}$. The results from these videos showed that the average speed of the nauplii at five different replicates remained at the same level for 24 h (Table 3). As a result, the movement of the nauplii is not affected by time in controls. This experiment was repeated for every experiment and always the results verified that the mobility of the nauplii remained constant after 24 h. Consequently, the movement of the nauplii was affected only from the existence of toxic compounds.

Table 3. Mobility of *Artemia salina* nauplii in ASW at $time_0$ and $time_{24}$.

Time (h)	Speed (m/s) per nauplius
0	0.011444
24	0.011636

The 24-h EC_{50} of potassium dichromate was estimated to 36.0 mg/L by the best fit model which is within the limits proposed by Svensson *et al*. [6].

The toxic effect of Cd^{2+} was obvious between 10^2 mg/L where the nauplii retained the 100% of their mobility and 10^3 mg/L where the nauplii had 25% of the initial mobility (Figure 1). The LC_{50} was estimated at 710.7 mg/L. The mobility of the nauplii, as evaluated by the camera-based instrument, had better sensitivity since the cadmium toxic effect was detectable at the concentration of 10 mg/L (100% mobility). The EC_{50} was 54.8 mg/L (Table 4), which is 13 times lower than the LC_{50} value. Kissa and co-workers [18] have estimated the 48 h LC_{50} at 159.6 mg/L. Furthermore, the experimental measurements were significantly different from the hypothetical values according to the t-test performed ($p < 0.05$).

The nauplii were not affected at the concentrations of 10^{-4}–10^{-1} mg/L and retained 100% of their mobility according to the hypothetical curve in Cu^{2+} samples. Deterioration of the mobility was observed between 10^{-4} mg/L and 10^2 mg/L, 84.1% and 10.9% mobility respectively by the camera-based device (Figure 1). The greater sensitivity allowed the detection of toxic levels even in very low concentrations. Furthermore, the EC_{50} value (7.6 mg/L) (Table 4) was 2.5 times lower than the LC_{50} (19.5 mg/L) and 1.3 times lower than the estimated 24 h LC_{50} of copper (9.5 mg/L) according to Gajbhiye and Hirota [4]. For this toxicant, the camera-based instrument proved more sensitive than the data in the literature and much more accurate than the mortality assay results.

The narrow range of 10^2 mg/L and 10^3 mg/L of Fe^{2+} proved toxic to *Artemia salina* nauplii according to the hypothetical curve. The LC_{50} could not be estimated due to extreme results. However, the lowest concentration detected as being toxic using the camera-based instrument was 10^{-4} mg/L Fe^{2+}, which corresponds to 79.4% of their mobility (Figure 1). The EC_{50} was estimated at 66.3 mg/L (Table 4). The sensitivity range was extended 10^6 times by the camera-based instrument comparing to the sensitivity range of the hypothetical curve. Although the 24-h EC_{50} was higher than the value

of 18.2 mg/L determined by Gajbhiye and Hirota [4], the detection range was greater with the camera-based device.

The hypothetical values achieved showed that the *Artemia salina* nauplii were sensitive to Zn^{2+} at 10^2 mg/L and 10^3 mg/L with 100% and 50% of their initial mobility respectively. By using the camera-based instrument higher sensitivity was achieved. The obtained curve presented a more constant trend with significantly different results from the hypothetical values according to the t-test performed ($p < 0.05$). The highest value of percentage mobility was observed at 10^{-2} mg/L corresponded to 88% mobility. The detection of toxic compounds proved successful at very low concentrations (Figure 1). The EC_{50} was 12.4 times lower (80.5 mg/L) than the LC_{50} value (1,000 mg/L) (Table 4). Gajbhiye and Hirota [4] evaluated the LC_{50} for zinc to 17.8 mg/L on *Artemia* nauplii. The high LC_{50} value might be due to the small population of shrimps used which lead to the increase of the

mortal probability.

Table 4. The LC_{50} and EC_{50} values of the tested metals in mg/L as measured for the mortality assay (probit analysis) and camera-based instrument (best fit model).

Substance	LC_{50} (mg/L)	EC_{50} (mg/L) (±SD)
Cd^{2+}	710.7	54.8 ± 11.1
Cu^{2+}	19.5	7.6 ± 8.3
Fe^{2+}	NE*	66.3 ± 9.7
Zn^{2+}	1,000.0	80.5 ± 17.1

*NE: Not estimated.

Earlier studies on the toxic effect of heavy metals on the mortality of *Artemia* species have used very limited ranges in order the LC_{50} to be specified. For example, Kissa *et al*. [18] have used a range of 1 to 200 mg/L. Gajbhiye and Hirota [4] have utilised different ranges dependent on the metal with a minimum of 0.1 mg/L and a maximum of 100 mg/L. Venkateswara *et al*. [19], who have studied the toxic effect of organophosphates on lethality of *Artemia salina*, also used a limited range of 0.2 mg/L to 3,000 mg/L dependent again on the tested toxic compound. In this study the EC_{50} values calculated with high sensitivity by using more extended scales. Moreover, *Artemia salina* species have been referred as organisms that accumulate toxic compounds with no effect on their life cycle. More specifically, it has been reported by Sarabia *et al*. [20] that *Artemia* species are very tolerant to

cadmium exposure as mentioned before with LC_{50} values varied from 93.3 to 280 mg/L. On the contrary, this study has proved that the accumulation of toxic compounds has acute effects on the mobility of the nauplii after 24 h of exposure to the toxicants and this is measurable. This research was performed for quantifying the mobility of the nauplii in very low concentrations, which normally showed no affects in other studies. According to the results achieved this was successfully performed and the system showed high sensitivity detection levels extended to concentrations of parts per trillion.

The software specifications have also minimised the possible errors such as the colour of the solution and the particulate settling to the minimum (Figure 3).

Figure 3. The first image shows the solution of 1,000 mg/L Zinc, the second one the iron solution of 1,000 mg/L and the third one Cadmium solution of 500 mg/L.
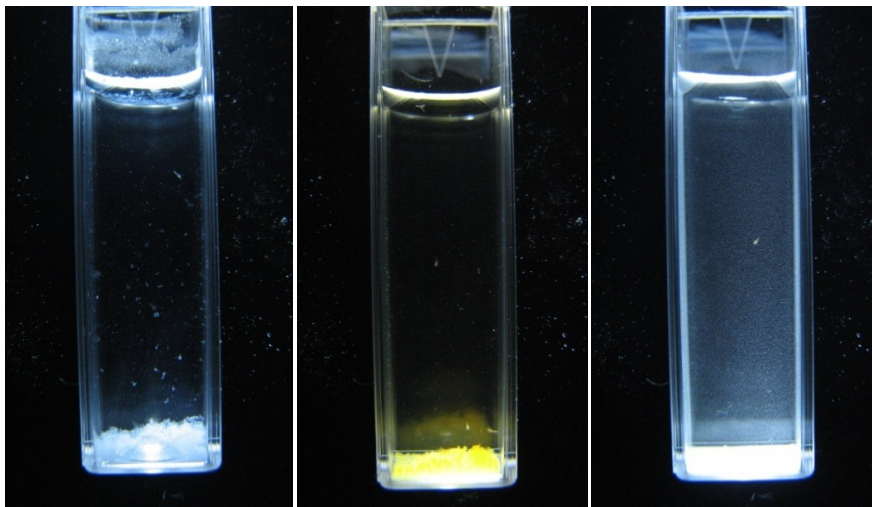
Finally, the difference between the LC_{50} and EC_{50} values was noticeable in our experimental data. This happened due to the small populations used in the experiments. Using only four nauplii for assessing the mortality in toxic solutions showed very ambiguous results comparing to the results in the bibliography (Gajbhiye and Hirota [4]; Kissa *et al*. [18]) where the researchers used at least ten nauplii for each sample. In contrast, the mobility of the *Artemia salina* nauplii could be estimated with good sensitivity using the camera-based device even when the population is very low. Consequently, the calculation of the EC_{50} values even with small number of nauplii proved possible.

4. Conclusions

The results obtained demonstrate the ability of the system to accurately detect water toxicity in a short period of time and at low cost. The detection of toxic compounds was achieved in very early stages by estimating the mobility of the nauplii in toxic aqueous solutions and compared to the mobility of the organisms in control samples. The camera-based device can provide information about toxic compounds (including metals such as Cu, Cd, Fe and Zn) at a wide range of concentrations
from 10^{-4} mg/L to 10^{3} mg/L. As a toxicity assay this approach is significantly more sensitive than the mortality-based assays, which can only detect concentrations starting from 10^{-1} mg/L. Furthermore, this study has proved that the accumulation of toxic compounds to *Artemia salina* nauplii has a deteriorating effect on their mobility. This deterioration was measured with high sensitivity by

tracking the nauplii and estimating their average speed using digital image processing. Possible errors of the system achieved to be minimized and more accurate results to be obtained. The most of the findings achieved in this study have been verified from the literature and this leads to a reliable system with high sensitivity in low cost. Thus, this novel approach constitutes a more affordable instrument for promptly detecting toxic substances in aquatic solutions.

References

1. Giarratano, E.; Comoglio, L.; Amin, O. Heavy metal toxicity in Exosphaeroma gigas (Crustacea, Isopoda) from the coastal zone of Beagle Channel. *Ecotoxicol. Environ. Safety* **2007**, *68*, 451-462.

2. Lorenzon, S.; Francese, M.; Ferrero, E.A. Heavy metal toxicity and differential effects on the hyperglycemic stress response in the shrimp Palaemon elegans. *Arch. Environ. Contam. Toxicol*. **2000**, *39*, 167-176.

3. MacRae, T.H.; Pandey, A.S. Effects of metals on early life stages of the brine shrimp, *Artemia*: A developmental toxicity assay. *Arch. Environ. Contam. Toxicol*. **1991**, *20*, 247-252.

4. Gajbhiye, S.N.; Hirota, R. Toxicity of heavy metals to brine shrimp *Artemia*. *J. Indian Fish. Assoc.* **1990**, *20*, 43-50.

5. Barahona, M.V.; Sánchez-Fortún, S. Comparative sensitivity of three age classes of *Artemia salina* larvae to several phenolic compounds. *Bull. Environ. Contam. Toxicol*. **1996**, *56*, 271-278.

6. Svensson, B.; Mathiasson, L.; Mårtensson, L.; Bergström, S. *Artemia salina* as test organism for assessment of acute toxicity of leachate water from landfills. *Environ. Monit. Assess*. **2005**, *102*, 309-321.

7. Pelka, M.; Danzl, C.; Distler, W.; Petschelt, A. A new screening test for toxicity testing of dental materials. *J. Dentistry* **2000**, *28*, 341-345.

8. Harwig, J.; Scott, P.M. Brine Shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. *Appl. Environ. Microbiol.* **1971**, *21*, 1011-1016.

9. Koutsaftis, A.; Aoyama, I. Toxicity of four antifouling biocides and their mixtures on the brine shrimp *Artemia salina*. *Sci. Total Envir*. **2007**, *387*, 166-174.

10. Nunes, B.S.; Carvalho, F.D.; Guilhermino, L.M.; Van Stappen, G. Use of the genus *Artemia* in ecotoxicity testing. *Environ. Pollut*. **2006**, *144*, 453-462.

11. Vanhaecke, P.; Persoone, G.; Claus, C.; Sorgeloos, P. Proposal for a short-term toxicity test with *Artemia* nauplii. *Ecotoxicol. Environ. Safety* **1981**, *5*, 382-387.

12. Vanhaecke, P.; Persoone, G. The ARC-test: A standardized short-term routine toxicity test with *Artemia* nauplii: Methodology and evaluation. In *Ecotoxicological Testing for the Marine Environment*; Persoone, G., Jaspers, E., Claus, C., Eds.; State University of Ghent, Institute for Marine Science Research: Bredene, Belgium, 1984; Volume 2, pp. 143-157.

13. Varó, I.; Serrano, R.; Pitarch, E.; Amat, F.; Lopez, F.J.; Navarro, J.C. Bioaccumulation of chlorpyrifos through an experimental food chain: Study of protein HSP70 as biomarker of sublethal stress in fish. *Arch. Environ. Contam. Toxicol*. **2002**, *42*, 229-235.

14. Varó, I.; Navarro, J.C.; Amat, F.; Guilhermino, L. Characterisation of cholinesterases and evaluation of the inhibitory potential of chlorpyrifos and dichlorvos to *Artemia salina* and *Artemia parthenogenetica*. *Chemosphere* **2002**, *48*, 563-569.

15. Vanhaecke, P.; Persoone, G.; Claus, C.; Sorgeloos, P. Research on the development of a standard toxicity test with *Artemia* nauplii. In *The Brine Shrimp Artemia*; Persoone, G., Sorgeloos, P., Roels, O., Jaspers, E., Eds.; Universa Press: Wetteren, Belgium, 1980; Volume 1, pp. 263-285.

16. Togulga, M. The short-term toxicity of two toxicants to *Artemia* nauplii. *Turkish J. Zool.* **1998**, *22*, 259-266.

17. Portmann, R.; Leumann, M.; Thommen, S. Method and Device for Determining Toxicity as Well as the Use Thereof, WO95/25955. U.S. Patent 5,789,242, 4 August 1998.

18. Kissa, E.; Moraitou-Apostolopoulou, M.; Kiortsis, V. Effects of four heavy metals on survival on hatching rate of *Artemia salina* (L.). *Arch. Hydrobiol.* **1984**, *102*, 255-264.

19. Venkateswara, R.J.; Kavitha, P.; Jakka, N.M.; Sridhar, V.; Usman, P.K. Toxicity of organophosphates on morphology and locomotor behavior in brine shrimp, *Artemia salina*. *Arch. Environ. Contam. Toxicol*. **2007**, *53*, 227-232.

20. Sarabia, R.; Del Ramo, J.; Varó, I.; Díaz-Mayans, J.; Torreblanca, A. Comparing the acute response to cadmium toxicity of nauplii from different populations of *Artemia*. *Environ. Toxicol. Chem.* **2002**, *21*, 437-444.

Cranfield Health

Effect of low temperatures on the Electrochemical Peroxidation treated samples and on their toxicity

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Introduction

Electrochemical Peroxidation (ECP) was used to treat compost leachate samples in order to make them suitable for the drainage system. Plant cells (chloroplasts) were then applied to measure their toxicity. During ECP, ferrous ions are produced from mild steel electrodes and react with hydrogen peroxide which has been injected into the system. The produced hydroxyl radicals oxidise the organic matter existing in the leachate. Samples were taken before the treatment starts and during the process and stored for up to 3 days at 4°C. The chloroplast assay was applied, firstly, to some toxic compounds (atrazine, phenol, sodium hypochlorite and copper nitrate) for validation purposes and then to the leachate samples. The effect of low temperatures was examined (a) on the colour and (b) on the toxicity of the untreated and ECP treated samples.

Methods/Materials

- 6L compost leachate (pH 4) were treated / run.
- Injection of 25mL H_2O_2 through the 'Y'-piece.
- Samples: before treatment and after 5, 7.5, 10, 15, 20, 30 and 40 minutes
- Voltage: 2.5-3V - Switching rate: 5 cycles /min
- Flow rate: 1.5L / min
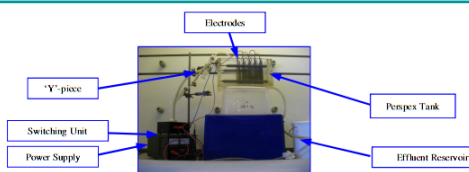
- Two extraction buffers were used:
  1. Extraction buffer (pH 8)
     [0.35M Sucrose, 50mM Tris-HCl (pH 8) and 10mM NaCl]
  2. Extraction buffer with 1% BSA

1. 30μL of chloroplast extraction diluted in the extraction buffer (1:9);
2. 40μL of either water (control) or sample;
3. 100μL of 0.6mM DPIP in 50mM Tris-HCl buffer, pH 8.
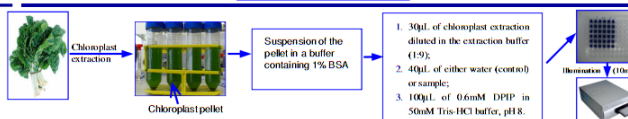
Results

- Great difference in colour and clarity was observed after the storage period of 3 days. Low temperature of 4°C helped particulate settling and the samples turned virtually colourless (Figs 1 & 2).

- Atrazine, at high concentrations (Fig 3), inhibits the Hill reaction (eq.1) which means that it is toxic to plants.

$$H_2O + DPIP_{ox} \xrightarrow[hv]{Chloroplasts} DPIP_{red} + 1/2O_2 \quad (eq.1)$$

- Copper nitrate induced a moderate response at the highest concentration tested (10^{-4}M), sodium hypochlorite and phenol proved to be far less toxic to chloroplasts.
- An inconstant rate of toxicity was observed for both parts of the samples (Fig 4). However, the absorbance values and consequently the observed toxicity were generally lower for the samples stored for 3 days in the fridge.

Figure 1. Samples before storage.
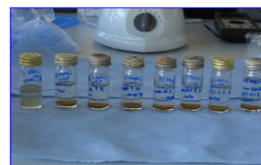
Figure 2. Samples after 3 days of storage at 4°C.
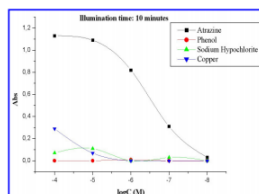
Figure 3. Chloroplast assay for the representative compounds.
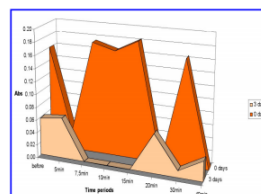
Figure 4. Effect of storage time (3 days, 4°C) on toxicity of the compost leachate samples.

Conclusions

Low temperatures led to better settling of the compounds existing after the ECP process. Additionally, the toxicity ranged in lower scale after 3 days at 4°C. In comparison to atrazine toxic levels, which was the most toxic compound of the four, the treated and even the untreated samples were not toxic. Further toxicity assays need to be applied and chemical analysis is required to support these results.

References

- Paton, I.K., Lemon M., Freeman B. and Newman J.D. (2009), "Electrochemical peroxidation of contaminated aqueous leachate", *Journal of Applied Electrochemistry*, pp. 1-4 (Article in press).
- Pratap, K. and Lemley, A. T. (1998), "Fenton Electrochemical Treatment of Aqueous Atrazine and Metolachlor", *Journal of Agricultural and Food Chemistry*, vol. 46, no. 8, pp. 3285-3291.
- Scrudato, R., Chiarenzelli, J. (2000). Electrochemical peroxidation of contaminated liquids and slurries. WO 00/17113, United States Patent 6045707.

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Effect of low temperatures on the Electrochemical peroxidation treated samples and on their toxicity

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Detecting Water Toxicity Using Digital Image Processing

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Abstract

This study addresses the problem of detecting toxic substances in water by using digital image processing. Specifically, a novel approach is presented for measuring the change in mobility of crustaceans when they are exposed to toxic compounds. Several state-of-the-art techniques have studied the effect of water toxicity to crustaceans by measuring the mortality rate, hatchability of cysts, rate of oxygen consumption or enzyme disruptions. This work studies the acute toxic effect of copper, cadmium, iron and zinc to *Artemia salina* and proves that their ability to move is measurable. A camera-based instrument was used to track the movement of these crustaceans and calculate the deterioration in their mobility. The results demonstrate that concentrations of parts per trillion of toxic compounds could be detected. These results should have significant applications for promptly identifying the presence of toxic substances in water at low cost.

INTRODUCTION

The ongoing interest in assessing the environmental effect of toxic waste materials has lead to an increasing number of bioassays and screening devices. Such techniques are often based on measuring the effect of toxic substances on the behaviour and life cycle of microorganisms, invertebrates and algae. Different biological properties that have been utilized for toxicity screening include the luminescence of *Vibrio fischeri*, respiratory rate of aerobic bacteria, enzyme activity of *Dapnia magna*, fluorescence of *Chlorella vulgari (algae)* and the lethality of crustaceans (such as *Artemia salina*). In addition, several studies have specifically focused on the impact of metals to the biota, by analyzing their toxic effect to crustaceans. Giarratano et al. (2007) and Lorenzon et al. (2000) have investigated the effect of heavy metals to *Exosphaeroma gigas* and *Palaemon elegans* respectively. Furthermore, MacRae and Pandey (1991) have researched the relation between water toxicity and hatching success of Artemia species. Similarly, Gajbhiye and Hirota (1990) have proved that the lethality of these species is dependent on the concentration of heavy metals in water.

Artemia species, or brine shrimps, have also been used in many scientific experiments for acute toxicity testing of toxic materials including pesticides (Baharona & Sanchez-Fortun, 1999), leachates (Svensson et al., 2005), dental materials (Pelka et al., 2000), fungal toxins (Harwig & Scott, 1971) and antifouling biocides (Koutsaftis & Aoyama, 2007). Although *Artemia* species are not considered as sensitive as other screening instruments or organisms (Nunes et al., 2006), they have some important advantages (Vanhaecke at al., 1981) including constant commercial availability all year round, cost-efficient, easy to culture, short life-cycle, no feeding required during the assay and great offspring production.

These advantages have led to a wide range of *Artemia*-based bioassays. The determination of the LD_{50} of the nauplii (instar II-III stage) (Vanhaecke & Persoone, 1984), the hatchability of the cysts (MacRae & Pandey, 1991), the different age specimens (Barahona and Sánchez-Fortún, 1996) and the disruptions on an enzyme property (Varó et al., 2002a, b) are only some of the *Artemia* end-points that have already been examined as evidence of toxicity. Specifically, Vanhaecke and Persoone (1984) have demonstrated a shrimp-based bioassay, known as the ARC-test (Artemia Reference Centre), with highly reproducible and accurate results. Additionally, Vanhaecke et al. (1980) proved that the most sensitive age for the majority of the tested compounds were the 48-hours old nauplii at the stage of instar II-III. The same results have also been supported by Barahona and Sánchez-Fortún (1996) and Toğulga (1998). On the other hand, *Artemia* species are very resistant to metals and accumulate them without any obvious effect on their life-cycle (Sarabia et al., 2006).

This study is based on a patented device by Portmann et al. (1998), which has been redesigned to be more accurate in recording small changes in the mobility of *Artemia salina* nauplii using a monocular camera and a digital image processing algorithm. The main hypothesis of this work was that the accumulation of toxic compounds to *Artemia* organism has a deteriorating effect on its mobility and this deterioration can be measured. Potassium dichromate was firstly tested as a reference

substance. Subsequently, copper, cadmium, iron and zinc were tested for their mortal effect on *Artemia* nauplii. Furthermore, the survivors were also counted and their hypothetical mobility was estimated. Finally, the mobility of the shrimps in those toxic metal samples was recorded and compared to the hypothetical one. The results showed that concentrations of parts per trillion of toxic metals affected the mobility of the shrimps and these changes could be detected consistently using the proposed technique.

MATERIALS AND METHODS

Preparation of the test organisms

Artemia cysts (San Francisco Strain Brine shrimps) were incubated in artificial seawater (ASW) of 3.5% salinity, at 30°C and pH 8.0 ± 0.5 and under constant aeration for 48 hours.

Test chemicals

The reagents were of general laboratory grade unless stated otherwise. Potassium dichromate was used as reference substance and was provided from BDH (Lutterworth, UK). Copper sulphate [$CuSO_4$ $5H_2O$] and cadmium chloride [$CdCl_2$ xH_2O] were obtained from Sigma-Aldrich (Gillingham, UK). Copper nitrate [$Cu(NO_3)_2$ $3H_2O$] was provided from Fisher Scientific (Loughborough, UK). Zinc sulphate [$ZnSO_4$ $7H_2O$] and iron sulphate [$FeSO_4$ $7H_2O$] were obtained from Acros Organics (Loughborough, UK). Stock solutions of 10000mg/L of all reagents were prepared in distilled water and then serial dilutions in artificial seawater were prepared for each metal.

Further Equipment

A camera (Canon Power shot S3 IS) and a laptop were used to track the movement of the shrimps by recording their activity for two minutes. Subsequently, the captured video sequence was analysed by a digital image processing algorithm that estimates the mobility of the shrimps. The concept has been based on the U.S. Patent of Portmann et al. (1998).

Mortality assay

The *Artemia* cysts were cultured in ASW until the stage of instar II-III nauplii (48 hours old). Four mL of either ASW (controls) or the tested compound was added to each cell. Then, four shrimps were transferred with Pasteur pipette with the minimal of ASW carried over. Five replicates were performed for the controls and every toxicant dilution. After 24 hours of incubation, the dead shrimps were counted and the LD_{50} values estimated with probit analysis. The assay would be considered valid if the mortality percentage of the control does not exceed the 10% (Vanhaecke et

al., 1981) and if the estimated LD_{50} for the reference substance (potassium dichromate) ranges between 30 and 50mg/L (Svensson et al., 2005).

Hypothetical mobility of the shrimps

The main advantage of the proposed approach is that it can quantitatively measure subtle changes in the mobility of shrimps and as a result detect small concentrations of toxic substances. This approach essentially makes the assumption that alive shrimps in the toxic samples have retained 100% of their initial mobility in the controls. In Figure 1, the hypothetical mobility is plotted against the actual mobility of the shrimps in order to compare the proposed methodology to the mortality assay. In the hypothetical mobility curve each value is equal to the number of alive shrimps multiplied by the average mobility per shrimp in the controls. The average mobility per shrimp is calculated by the digital image processing algorithm.

Mobility assay using digital image processing

Video sequences were recorded for two minutes for all the controls and samples. In each video sequence, the shrimps were counted and tracked as illustrated in Figure 2. Subsequently, the average speed of each shrimp was derived by dividing the length of the tracking path by the recording time. The Effective Concentration (EC_{50}) was estimated through the «best fit model» (Excel, Microsoft Office, 2007). The best fit model revealed that the most appropriate equation was the quadratic equation.

Statistical analysis

Statistical analysis of one-way ANOVA of the data was performed using Genstat v.12.0 (VSN International Ltd, Hemel Hempstead, UK) and was evaluated at the $p<0.001$ level, as presented in Tables 2, 3, 4, and 5. Furthermore, the 24 hours LD_{50} was determined by using Biostat v.5.2.5.0 (AnalystSoft, 2008).

RESULTS AND DISCUSSION

The results from the mortality assay of *Artemia* nauplii at the stage of instar II-III were considered valid since the mortality percentage of the controls was 0%. The 24-hour LD_{50} of potassium dichromate was estimated to 84.2mg/L with probit analysis and EC_{50} value to 40.0mg/L by the best fit model. Vanhaecke et al. (1980) has estimated the 24-hour LC_{50} of the nauplii between the values of 27.9 and 39mg/L and Toğulga (1998) from 32 to 42mg/L.

All the curves from the tested metals achieved by the camera-based instrument, presented a statistically significant decrease as the concentration increased and presented in the Figure 1. The LD_{50} and EC_{50} values are listed in the Table 1.
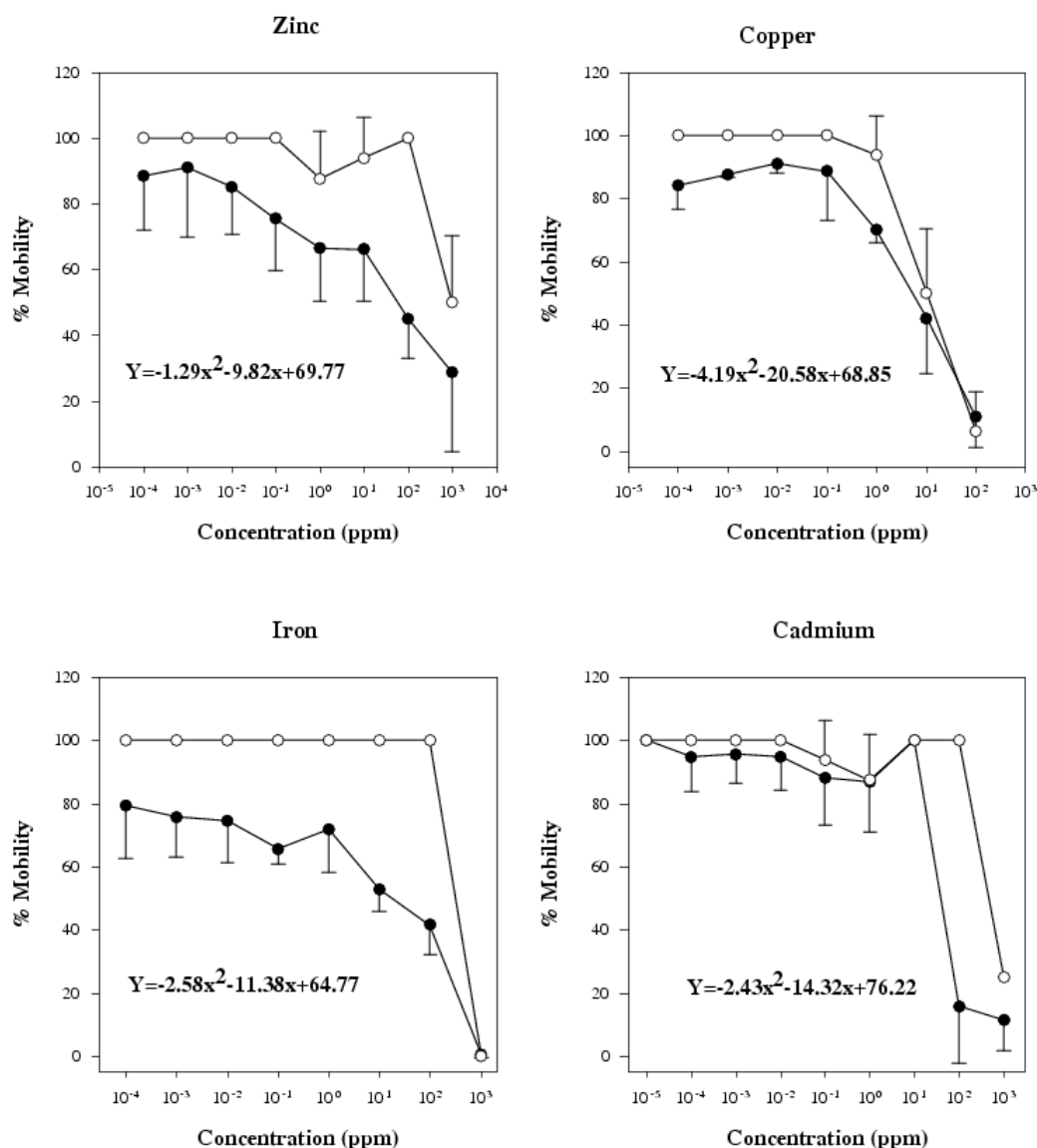
Figure 1. Percentage mobility of (o) hypothetical and (●) camera-based monitored curves of the *Artemia* nauplii for zinc, copper, iron and cadmium. The equations correspond to the camera-based curves trend. The error bars represent the standard error from the four replicates for each concentration.

The hypothetical values achieved showed that the *Artemia* nauplii were sensitive to Zn^{2+} at 10^{-1} mg/L and 10^3 mg/L with 100% and 50% of their initial mobility respectively. By using the camera-based instrument higher accuracy was achieved. The obtained curve presented a more constant trend. The highest value of percentage mobility was observed at 10^{-4} mg/L or 10^2 ng/L corresponded to 88.5%

mobility. The detection of toxic compounds proved successful at very low concentrations. The EC_{50} was 19.5 times lower (44.7mg/L) than the LD_{50} value (1000mg/L). Gajbhiye and Hirota (1990) evaluated the LD_{50} for zinc to 17.8mg/L on *Artemia* nauplii. The extremely high LD_{50} value might be due to the small population of shrimps used which lead to the increase of the mortal probability.

Figure 2. The left image shows a representative video frame as captured by the camera. A sequence of such frames forms the input to the digital image processing algorithm. Although the shrimps are not clearly visible in the cuvette, they can be tracked with good accuracy in order to measure their mobility. The remaining images show some characteristic examples of tracking paths which were derived over 6.6 sec intervals. Lighter gray shades denote areas of higher mobility including overlapping tracking paths.

The nauplii were not affected at the concentrations of 10^{-4}-10^{-1}mg/L and retained 100% of their mobility according to the hypothetical curve in Cu^{2+} samples. Deterioration of the mobility was observed between 10^{-4}mg/L and 10^{2}mg/L, 84.1% and 10.9% mobility respectively by the camera-based device. The greater sensitivity allowed the detection of toxic levels even in very low concentrations. Furthermore, the EC_{50} value (6.2mg/L) was 3 times lower than the LD_{50} (19.5mg/L) and 1.5 times lower than the estimated 24 hour LD_{50} of copper (9.5mg/L) according to Gajbhiye and Hirota (1990). For this toxicant, the camera-based instrument proved more sensitive than the data in the literature and much more accurate than the mortality assay results.

The limited range of 10^{2}mg/L and 10^{3}mg/L of Fe^{2+} proved toxic to brine shrimps according to the hypothetical curve. The LD_{50} could not be estimated due to the extreme results. The lowest concentration detected as toxic from the camera-based instrument was 10^{2} ng/L Fe^{2+}, which corresponds to 79.4% of their mobility. The EC_{50} was estimated at 11.2mg/L. The sensitivity range was extended 10^{6} times by the camera-based instrument comparing to the sensitivity range of the

hypothetical curve. Additionally, the 24 hour EC_{50} was 1.6 times lower than the value of 18.2mg/L determined by Gajbhiye and Hirota (1990).

The toxic effect of Cd^{2+} was obvious between 10^{-2}mg/L where the nauplii retained the 100% of their mobility and 10^{3}mg/L where the nauplii had 25% of the initial mobility. The LD_{50} was estimated at 710.7mg/L. The mobility of the nauplii, as evaluated by the camera-based instrument, had better sensitivity since the cadmium toxic effect was detectable at the concentration of 10^{2}ng/L (94.7% mobility). The EC_{50} was 29.5mg/L which is 17times lower than the LD_{50} value. Kissa and co-workers (1984) have estimated the 48hours LC_{50} at 159.6mg/L.

Table 1. The LD_{50} and EC_{50} values of the tested metals in mg/L as measured for the mortality assay (probit analysis) and camera-based instrument (best fitting model).

Substance	LD_{50} (mg/L)	EC_{50} (mg/L)
Zn^{2+}	1000.0	44.7 ± 2.2
Cu^{2+}	19.5	6.2 ± 2.1
Fe^{2+}	NE*	11.2 ± 5.9
Cd^{2+}	710.7	29.5 ± 9.8

*NE: Not estimated

Earlier studies on the toxic effect of heavy metals on the mortality of *Artemia* species have used very limited scales. For example, Kissa et al. (1984) have used a range of 1 to 200mg/L. Gajbhiye and Hirota (1990) have utilised different ranges dependent on the metal with a minimum of 0.1mg/L and a maximum of 100mg/L. Venkateswara et al. (2007), who have studied the toxic effect of organophosphates on lethality of *Artemia salina*, also used a limited range of 0.2mg/L to 3000mg/L dependent again on the tested toxic compound. Thus, the achievement of this study is that the camera-based instrument successfully extended the detection range of toxicity to parts per trillion (ng/L).

Table 2. One-way ANOVA table of the mobility of the shrimps monitored in cadmium solutions.

Source of variation	Degrees of freedom	Sums of squares	Mean square	Variance ratio	F probability
Concentrations	7	39793.7	5684.8	37.50	<0.001
Residual	23	3486.3	151.6		
Total	30	39033.4			

Table 3. One-way ANOVA table of the mobility of the shrimps monitored in copper solutions.

Source of variation	Degrees of freedom	Sums of squares	Mean square	Variance ratio	F probability
Concentrations	6	22153.0	3692.2	30.71	<0.001
Residual	18	2163.9	120.2		
Total	24	22840.3			

Table 4. One-way ANOVA table of the mobility of the shrimps monitored in iron solutions.

Source of variation	Degrees of freedom	Sums of squares	Mean square	Variance ratio	F probability
Concentrations	7	19541.1	2791.6	23.61	<0.001
Residual	24	2838.0	118.2		
Total	31	22379.1			

Table 5. One-way ANOVA table of the mobility of the shrimps monitored in zinc solutions.

Source of variation	Degrees of freedom	Sums of squares	Mean square	Variance ratio	F probability
Concentrations	7	13469.5	1924.2	7.82	<0.001
Residual	21	5166.1	246.0		
Total	28	17653.0			

Moreover, *Artemia salina* species have been referred as organisms that accumulate toxic compounds with no effect on their life cycle. More specifically, it has been reported by Sarabia et al. (2002) that Artemia species are very tolerant to cadmium exposure as mentioned before with LC_{50} values varied from 93.3 to 280mg/L. On the contrary, this study has proved that the accumulation of toxic compounds has effects on the mobility of the nauplii and this is measurable.

Finally, the difference between the LD_{50} and EC_{50} values was noticeable in our experimental data. This happened due to the small populations used in the experiments. Using only four shrimps for assessing their mortality in toxic solutions showed very ambiguous results comparing to the results in the bibliography (Gajbhiye and Hirota, 1990; Kissa et al. 1984) where the researchers used at least ten shrimps for each experiment. In contrast, the mobility of the Artemia nauplii could be estimated with good accuracy using the camera-based device even when the population was very low. Consequently, the number of shrimps in the camera-based device does not affect the estimation of toxicity and the EC_{50} values.

CONCLUSIONS

The results obtained demonstrate the ability of the system to accurately detect water toxicity in a short period of time and at low cost. The camera-based device can provide information about toxic compounds (including metals such as Cu, Cd, Fe and Zn) at a wide range of concentrations from 10^{-4} mg/L to 10^{3} mg/L. As a toxicity assay this approach is significantly more sensitive than the mortality-based assays, which can only detect concentrations starting from 10^{-1} mg/L. Furthermore, this study has proved that the accumulation of toxic compounds to *Artemia* nauplii has a deteriorating effect on their mobility. This deterioration was measured by tracking the shrimps and estimating their average speed using digital image processing. Additionally, the performance of this technique was independent of the shrimp population, leading to a less complex and quicker assay. Thus, this novel approach can lead to more affordable instruments for promptly detecting toxic substances in aquatic solutions.

REFERENCES

Barahona M.V. and Sánchez-Fortún S. (1996). Comparative Sensitivity of Three Age Classes of Artemia salina Larvae to Several Phenolic Compounds. *Bulletin of Environmental Contamination and Toxicology*, **56**(2), 271-278.

Gajbhiye S.N. and Hirota R. (1990). Toxicity of heavy metals to brine shrimp Artemia. *Journal of the Indian fisheries Association*, **20**, 43-50

Giarratano E., Comoglio L. and Amin O. (2007). Heavy metal toxicity in Exosphaeroma gigas (Crustacea, Isopoda) from the coastal zone of Beagle Channel. *Ecotoxicology and environmental safety,* **68**(3), 451-462.

Harwig J and Scott P. M. (1971). Brine Shrimp (Artemia salina L.) Larvae as a Screening System for Fungal Toxins. *Applied and Environmental Microbiology,* **21**(6), 1011-1016.

Kissa E., Moraitou-Apostolopoulou M. and Kiortsis, V. (1984). Effects of four heavy metals on survival on hatching rate of Artemia salina (L.). *Archiv fur Hydrobiologie,* **102**(2), 255-264.

Koutsaftis A. and Aoyama I. (2007). Toxicity of four antifouling biocides and their mixtures on the brine shrimp Artemia salina. *Science of the Total Environment,* **387**, 166-174.

Lorenzon S., Francese M. and Ferrero E. A. (2000). Heavy metal toxicity and differential effects on the hyperglycemic stress response in the shrimp Palaemon elegans. *Archives of Environmental Contamination and Toxicology,* **39**(2), 167-176.

MacRae T. H. and Pandey A. S. (1991). Effects of metals on early life stages of the brine shrimp, Artemia: A developmental toxicity assay. *Archives of Environmental Contamination and Toxicology,* **20**(2), 247-252.

Nunes B. S., Carvalho F. D., Guilhermino L. M. and Van Stappen G. (2006). Use of the genus Artemia in ecotoxicity testing. *Environmental Pollution,* **144**, 453-462.

Pelka M., Danzl C., Distler W. and Petschelt A. (2000). A new screening test for toxicity testing of dental materials. *Journal of dentistry,* **28**(5), 341-345.

Portmann R., Leumann M. and Thommen S. (1998). Method and device for determining toxicity as well as the use thereof, WO95/25955, United States Patent 5,789,242.

Sarabia R., Del Ramo J., Varo I., Díaz-Mayans J. and Torreblanca A. (2002). Comparing the acute response to cadmium toxicity of nauplii from different populations of Artemia. *Environmental Toxicology and Chemistry,* **21**(2), 437-444.

Svensson B., Mathiasson L., Mårtensson L. and Bergström S. (2005). Artemia salina as test organism for assessment of acute toxicity of leachate water from landfills. *Environmental monitoring and assessment,* **102**, 309-321.

Toğulga M. (1998). The Short-Term Toxicity of Two Toxicants to Artemia Nauplii. *Turkish Journal of Zoology,* **22**(3), 259-266.

Vanhaecke P., Persoone G., Claus C. and Sorgeloos P. (1980). Research on the development of a standard toxicity test with *Artemia* nauplii. In: The Brine Shrimp Artemia, Vol. 1, *Morphology, Genetics, Radiobiology, Toxicology* (G. Persoone, P. Sorgeloos, O. Roels and E. Jaspers, Eds.), 263-285. Universa Press, Wetteren, Belgium.

Venkateswara Rao J., Kavitha P., Jakka N. M., Sridhar V. and Usman P. K. (2007). Toxicity of organophosphates on morphology and locomotor behavior in brine shrimp, Artemia salina. *Archives of Environmental Contamination and Toxicology,* **53**(2), 227-232.

Vanhaecke P., Persoone G., Claus C. and Sorgeloos P. (1981). Proposal for a short-term toxicity test with Artemia nauplii. *Ecotoxicology and environmental safety,* **5**, (3), 382-387.

Vanhaecke P. and Persoone G. (1984). The ARC-test: a standardized short-term routine toxicity test with Artemia nauplii. Methodology and evaluation, 143-157. In: Ecotoxicological testing for the marine environment. Vol.2. Persoone G., Jaspers E. and Claus C. (Eds). State Univ. Ghent, Belgium; Inst. Mar. Sci. Res., Bredene, Belgium. 588.

Varó I., Navarro J.C. Amat F. and Guilhermino L. (2002). Characterisation of cholinesterases and evaluation of the inhibitory potential of chlorpyrifos and dichlorvos to Artemia salina and Artemia parthenogenetica. *Chemosphere*, **48**, 563-569

Varó I., Serrano R., Pitarch E., Amat F., Lopez F.J. and Navarro, J.C. (2002). Bioaccumulation of chlorpyrifos through an experimental food chain: study of protein HSP70 as biomarker of sublethal stress in fish. *Archives of Environmental Contamination and Toxicology*, **42**, 229-235.

Detecting water toxicity using digital image processing

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Development of a screening tool for early detection of contaminants in aqueous solutions using *Artemia salina*

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Abstract

This study aims to minimise the exposure time of *Artemia salina* nauplii to toxic aqueous solutions before measuring the effect to their mobility by using digital image processing. The instrument consists of a camera with a macro lens, a dark chamber, a light source and a laptop computer. The key principal of this method is the detection of subtle changes of shrimp mobility in toxic environment using advanced software. Four nauplii were inserted into a macro cuvette with contaminants such as copper, cadmium, zinc and iron ions and placed inside the dark chamber. Then, each sample was filmed for two minutes after sixteen, twenty and twenty-four hours of incubation and the videos were processed by a motion tracking algorithm that estimated their mobility. The results showed that there were no significantly different results between time intervals and concentrations for all compounds analysed. Although, the toxicity assays using *Artemia salina* allow at least 24 hours of exposure time before counting the dead larvae, this system successfully achieved to reduce this period in order to get prompt detection of contaminants.

Development of a screening tool for early detection of contaminants in aqueous solutions using *Artemia salina*

Annual Meeting 2010 of the Society of Environmental Toxicology and Chemistry UK,

Environmental Pollution in a Changing World

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Implementing a screening device for monitoring water toxicity

Postgraduate Conference

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Optimising a screening device for detecting and monitoring water toxicity

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Category: Monitoring Session

Abstract for poster

This study reports the optimisation of a screening device, which detects toxic compounds in aqueous solutions using *Artemia salina* (or brine shrimps) as test organism and digital image processing as part of the methodology. The instrument consists of a camera with a macro lens, a dark chamber, a light source and a laptop computer. The key principal of this method is the detection of subtle changes in the mobility of nauplii (48 hours old) in toxic environment using advanced software. Two experiments were performed. The aim of the first one was to determine the ideal temperature range where the nauplii retain a constant mobility level during a 24-hour-bioassay. The second experiment examined the optimum number of tested organisms by testing either four or eight nauplii in samples containing metal ions such as copper, cadmium, zinc and iron ions in various concentrations of total volume of 4mL. The experimental process followed, was to insert the nauplii into macro cuvettes with either artificial seawater or toxic solutions. Then, the cuvettes were placed inside the dark chamber and each sample was filmed for two minutes within 24 hours from incubation. At the end, the videos were processed by a motion tracking algorithm that estimated their mobility. For the first experiment, the results revealed that the nauplii retained their mobility at high levels between 15 and 25 °C. The results from the second experiment showed that the number of tested organisms does not significantly affect the accuracy of the instrument. In conclusion, this bioassay enables the accurate detection of low concentrations of toxic substances in aqueous solutions, while using small populations of shrimps.

Cranfield Health

Optimising a screening device for detecting and monitoring water toxicity
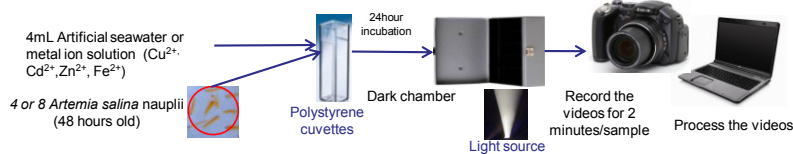
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Introduction

A screening device has been developed for detecting toxic compounds in aqueous solutions using Artemia salina (or brine shrimps) as a test organism and digital image processing as part of the methodology.

The **key principal** of this method is the detection of subtle changes in the mobility of *Artemia salina* nauplii in a toxic environment using advanced software.

The **aim of this study** was firstly, to determine the ideal temperature range where the nauplii retain their mobility for 24 hours and then, the optimum number of test organisms in samples containing metal ions such as copper, cadmium, zinc and iron.

Methods/Material

4mL Artificial seawater or metal ion solution $(Cu^{2+}, Cd^{2+}, Zn^{2+}, Fe^{2+})$

4 or 8 Artemia salina nauplii (48 hours old)

Polystyrene cuvettes

24hour incubation

Dark chamber

Light source

Record the videos for 2 minutes/sample
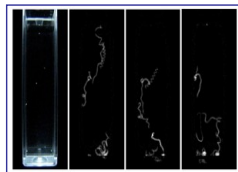
Process the videos

Results

Figure 1. The left image shows a representative video frame as captured by the camera. The remaining images show the characteristic tracking path of the nauplii.
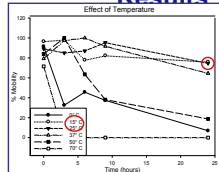
Figure 2. Effect of temperature on the mobility of the nauplii versus time
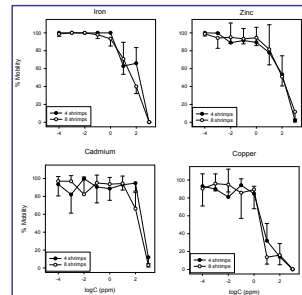
Figure 3. Effect of number of organisms in metal ion solutions on the mobility of the nauplii versus time

The mobility of the shrimps during 2 minutes was calculated.

If the environment is toxic then the movement of the nauplii deteriorates. *Artemia salina* nauplii retain their mobility in non-toxic solutions for 24 hours at temperatures between 15-25 °C.

"Critical range" determination was performed by testing geometrically spaced concentrations for all metal ions.

No significantly different results were obtained between the tests performed with 4 and 8 nauplii in all metal ion solutions. The results were also compared to the assay where counting of dead nauplii after 24 hours was performed in order to calculate the toxicity of a sample. The EC_{50} values determined with this device were the same or lower compared to the aforementioned test according to the literature (Gajbhiye and Hirota ,1990).

Conclusions

There is no need for temperature control inside the device since the shrimps retain their mobility between 15 °C and 25 °C.

The software used in this system offers high precision regardless of the number of test organisms inserted in each sample.

This screening tool consists an automated assay for detecting water toxicity at low cost.

References: Gajbhiye SN, Hirota R (1990) Toxicity of heavy metals to brine shrimp Artemia. Journal of the Indian fisheries Association 20:43-50

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Optimising a screening device for detecting and monitoring water toxicity

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