

The Tema Institute
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**THE EFFECTS OF HEAVY METALS ON DENITRIFICATION
IN A WETLAND SEDIMENT.**

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Titel

THE EFFECTS OF HEAVY METALS ON DENITRIFICATION IN WETLAND SEDIMENT.

Title

Författare

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Sammanfattning

Abstract:

Wetlands water quality is influenced by the anthropogenic activities in the catchments' areas. Wastewaters from the urban storm, agricultural runoff and sewage treatment often end up in wetlands before flowing to rivers, lakes and the sea. A lot of pollutants are readily transported in these wastewaters, thus subjecting the wetland ecosystem into a continuous resilience. Importantly, heavy metals like Cu, Zn, and Pb etc. are constituents of such pollutants in the wastewaters.

The study has as a specific objective to investigate the effects of heavy metal Cu, Zn and Pb on denitrification, an important ecosystem process and service. In a wetland situation, denitrification is a permanent nitrogen removal process accounting for about 90% of the total nitrogen removal.

The research was carried out in the laboratory and sediment samples were taken from a constructed wetland in Linköping. We employed acetylene inhibition technique in obtaining N₂O as a product resulting from nitrate reduction. The treatments (Cu, Zn and Pb) levels were 100 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg of sediment, in three replicates and a control.

Samplings of the assay were taken within 24hours. Gas chromatography was used to analyse and quantify N₂O in the various samples. A linear regression analysis was carried out with Windows Excel and SPSS to compare the various treatments with the control at 95% confidence level

The results show that there were no inhibitions of denitrification at 100 mg/kg sediment treatment level for any of the element. Inhibition of denitrification was observed at treatment levels 500 mg/kg and 1000 mg/kg of sediment .The rate of nitrate reduction was compared from the slope of the regression curve. The rate for Cu at 500 mg and 1000 mg /kg of sediment was moderately related to that of the control, Zn shows a similar trend but a higher rate in some samples, while Pb shows more inhibition

Nyckelord

Keywords : wetlands, heavy metals, inhibition, denitrification

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ABSTRACT

Wetlands water quality is influenced by the anthropogenic activities in the catchments' areas. Wastewaters from the urban storm, agricultural runoff and sewage treatment often end up in wetlands before flowing to rivers, lakes and the sea. A lot of pollutants are readily transported in these wastewaters, thus subjecting the wetland ecosystem into a continuous resilience. Importantly, heavy metals like Cu, Zn, and Pb etc. are constituents of such pollutants in the wastewaters.

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DEDICATION

To Lena and Stellan Nyden

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CHAPTER ONE

1.0 BACKGROUND INFORMATION

1.1 DEFINITIONS

Wetland definition lacks specifics, but generally it embraces three components which Gosselink and Mitsch (1993) indicated as: presence of water in the shallow surface of the soil or within the root level at most time of the year; a unique soil condition which differs from the upland and the presence of supportive water adaptive plants (Hydrophytes). Most definitions may encounter some problem of specifics with regard to the time and the duration of flooding, depth of the water, the size, endemic species, location and human impact implication.

Some definitions may try to address some of these problem areas. In terms of flooding wetlands could be seasonal or permanent. The depth is often described as shallow and the ecology is often seen as an edge of terrestrial and aquatic habitats, thus, being able to support facultative species (species that adapt to both wet and dry environments) and obligate species (i.e. adapted to aquatic conditions). Concerning the location and size, there is no definite size. The sizes range is often determined by the topography and the hydrology of the area. Wetlands could be located inland, close to coastal lakes, forests and savannahs. They could be fresh water or marine, and occur in rural or urban areas. Broadly, we have natural wetlands and artificial or constructed wetlands. The water quality of the wetland is a factor of the anthropogenic activities in the catchment area (Gosselink and Mitsch 1993).

To be effective, the scientific definition rendered by The US Fish and Wildlife Service defines wetlands as lands transitional between terrestrial and aquatic systems, where the water table is usually at or near the surface or the land is covered by shallow water, with any one of the characteristics such as the presence of undrained hydric soil substrate, predominant hydrophytes (at least periodically) and the non-soil substrate is water logged. (Cowardin et al 1979).

1.2 THE IMPORTANCE OF WETLAND TO THE ENVIRONMENT

The wetland by its unique position acts as a bridge between the land and the river or lake or coastal sea. It acts as the receiver of most pollutants generated from the anthropogenic activities taking place in the catchment basin.

The wetland helps to purify water either as wastewater from urban sewage treatment; storm water or run off from agricultural land, before the water gets into the water systems. It is the recognition of this vital natural process, that the application of constructed wetland for treatment of domestic and municipal wastewater, storm water and agricultural run off had gained much interest. Wetland removes suspended solids, carbonaceous biological oxygen demand; faecal coli form bacteria, certain heavy metals and phosphorus and nitrogen from wastewater (Gelt, 1997, Kadlec and Knight, 1996, and Hammer, 1989).

Wetland is an environment that supports numerous species of organisms, thus can be said to encourage biodiversity. It can also provide livelihood or economic support to communities in the area of fisheries and the supply of materials for local craft and tools. Some wetlands constitute a niche for many animal species such as fish: tilapia, catfish and shell fishes but also an excellent breeding ground especially in the coastal or river based wetlands. Wetland in some communities is useful in rice cultivation (swampy rice paddy). In the forest area it encourages the growth of palm trees and bamboo, which are used locally for construction and other economic uses (Mulugeta, 1994)

Wetland provides a suitable source of data in the area of environmental impact studies, because the water quality and the nature of the sediment are partly determined by the influence of the human activities and the nature of the basin catchments. The species fauna and flora responses in the wetland could be a good indicator of pollution impact, once base data are known. Wetland risk factors may include excessive load of pollutants, which could reduce the wetland's resilience or the capacity of the wetland to adjust itself. Examples are massive oil spillage either from burst pipes or broken down vessel tanks of which the oil could drift to the adjacent wetland. There are also the debalasting activities of the ships during occasional cleaning and oil spill could result from the used water from the oil wells

during oil drilling in many natural wetlands. (<http://oils.gpa.unep.org/facts/prevent-land.htm>).

Wetland sediment contaminants may include polycyclic aromatic hydrocarbon (PAHs), monocyclic hydrocarbon, pesticides, chlorinated hydrocarbons phthalates esters, metals, nutrients, suspended clay and others including cyanides and organo- metals (US EPA, 1993). Wetland hydrological morphology makes them most vulnerable as sinks for most pollutants

Development also imposed a lot of risk on wetland. The demand for more residential space, farmland, recreation and aesthetics of the environment had caused the drainage of most coastal wetlands reconstructed with human habitation. The consequence of this may include urban flooding, loss of biodiversity, coastal eutrophication etc. (FAO/ UNDP/UNEP, 1994)

Nutrient load in river, lake and coastal waters is of immense concern. The problem of coastal water eutrophication is high on the agenda for environmental policy of most global institutions like the European Union and the United Nation environmental programmes. The EU water directive specifies nitrate limit of 50 mg/L in drinking water. This applies to groundwater and surface water (Monteny 2001).

The task of reducing nitrate load flux to the surface and underground water makes wetland application become very relevant due to its efficiency in the removal of nitrogen from the wastewaters. Essentially, the process of nitrogen removal from the wastewater is a natural process called denitrification, which is a component of nitrogen cycle. It is a permanent removal of nitrogen from the nitrate form to gaseous N_2 or N_2O in the absence of oxygen (anaerobic condition). The denitrification process is facilitated naturally by bacterial activities, which are often moderated by the physiochemical conditions of the environment (Kadlec and Knight, 1996).

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 MATERIAL SINKS IN WETLAND

Wastewater effluents carry a range of materials in dissolved form and suspended solids, which are organic and inorganic. These materials are carried by urban storm water, sewage water from urban sewage treatment plant, run-off from agricultural field and waste from livestock yard, into wetlands, rivers, lakes and the sea (Chandler, 1994). Heavy metals ions constitute the material sinks in wetland. Heavy metals, which include mercury, cadmium, zinc, lead, chromium and copper, are used in most household facilities, pharmaceuticals, paint, battery, transportation etc. Investigation of waste generated from incinerators shows a number of materials in the ash enriched with heavy metals, in the fly ash or slag. About 80% of mercury (Hg) is from batteries, lead from waste oil used in some boilers. (Beasley and Kneale, 2001; United Nations- System wide Earth Watch, 2006).

The development of urban incinerator and central boiler systems in the emerging waste recycling strategy in environmental management may seem to open new areas of research interest. Wastes from such systems may no doubt get into the water system and to the wetland, where it become a sink. There is a global policy development with regards to heavy metals and hazardous substance emission, for instance, the EU water directive 2000 gave some specifics for drinking water quality with respect to heavy metal limits, lead 25 ug/L by 2003 and 10 ug/L by 2013(Water Framework Directive 2000/60/EC)

Wetlands also act as sinks to nutrients and sites where loads of nutrients are reduced from wastewater before such water enter the rivers or lakes. The microorganisms in a wetland situation are important in nutrient flux. Wastewater received in the wetland contains nutrients from urban sewage, agricultural waste and fertilizers, these nutrient loads are reduced by the activities of the: micro- organisms in the processes of nitrification and denitrification, wetlands plants uptake and assimilation, and sedimentation in the organic matters. The implication is that the wetland reduces the nutrient load of the wastewater, thus serving as a natural nutrient sink (Gosselink and Mitsch, 1993).

Nutrients abundant in water (especially nitrogen and phosphorous) cause eutrophication or a process of nutrient enrichment leading to the growth of algae or algal blooms. The algae decay following a bloom increase the BOD, causing fish stress and kill, and the death of benthic organisms (Nixon 1995; Kremser and Schung, 2002) Also nutrients are not desired in water due to drinking water status requirement for low nitrate levels. The concentration of unionized ammonia is also lethal to the fish. Thus, the process of nutrient removal in the aquatic system is very important for both human health and the sustainability of the aquatic ecosystem

Wetlands as a natural ecological design are important in water quality improvement as it receive water from the basin and enhance the quality status before discharge to the river ways. It is the understanding of this process that prompted the man made design of wetlands used in modern wastewater treatment. Hammer (1989) defines such innovation as constructed wetlands.

2.2 HEAVY METALS AND ENVIRONMENTAL RISK ISSUES

Heavy metals flux is a significant environmental health issues. Generally, metals in the right proportion are vital elements in the cells of organism and are important for healthy living. However excessive supply of these metals might become very fatal to health. Also, persistence exposure to low concentration of heavy metals constitutes a health risk (United Nations- System wide Earth Watch 2006).

Day (1998), reported on health problems associated with lead poisoning which include: neurological damages leading to secondary condition characterized by low intelligence, loss of short memory, poor coordination and learning disabilities. In a prenatal exposure, the condition may include low birth weight, poor immune responses or otherwise.

In adult, lead is suggested to have a linked to behavioural inhibition and even tooth decay (Masters, 1998; Gil et., al 1996). Mercury is also a significant health issue. High concentrations may cause nervous system damage, leading to deformities in early child exposure, partial blindness, muscles wasting, and loss of reflex. It is also associated with low: learning abilities, coordination, and fertilities in males (Dickman et al 1998).

Heavy metal like arsenic is said to cause cardiovascular disorder, skin-cancer and skin related diseases, kidney damage and peripheral neuropathy (WHO, 1997). Copper, which is a trace element needed in the body normal physiological performance, could cause anaemia, liver and kidney damages, stomach and intestinal problems when in excess concentration in the body.

Cadmium, which is another heavy metal, is closely associated with renal dysfunction, lungs cancer, bone abnormalities such as osteomalacia and osteoporosis on long -term exposure in humans and animals. It bio- accumulates in the liver and kidney and it could lead to kidney and liver failure (BFR, 2005).

Heavy metals could be a field pollutant in an agricultural land with a history of mining or related industrial activity. For example, cadmium is absorbed by crop plants roots along with plants nutrients; vegetables and fruits could be a link in the food chain. Also cadmium flux is reported to associate with cigarette smoke. In seafood e.g. fin and shellfishes from heavy metal polluted waters; the metals accumulate in the kidney and other organs and tissues of the fish, and when consumed, its bio- accumulates (BFR, 2005).

Heavy metal toxicity to microbial community often leads to microbial tolerance. Such tolerance is evidenced by an increase in the species dominance in the microbial community. Such environmental resistance or tolerance could reinforce into an environmental health risk. Bacterial tolerance to Cu, Zn, Ni and Pb was investigated by Frostegard et al. (1996). The results shown a marked tolerance of bacterial to copper, tolerance was also reported in Zn, Ni and Pb. It is suggested that microbial tolerance to heavy metals could be related to antibiotic resistance, as new strains of genes will build up in bacteria resistant in a polluted environment. Such genes resistant to chemicals may seem also to pose resistance to chemical drugs (Spain, 2003). There is therefore a justification for the research, which in a way want to examine the possible effects of heavy metal concentration on a wetland microbial process such as denitrification.

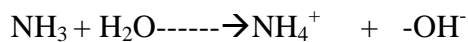
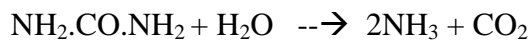
2.3 NITROGEN REMOVAL IN WETLANDS

Nitrogen cycling is a complex natural process where nitrogen transformation between organic, soluble inorganic and gaseous forms. In a wetland situation these three phases of nitrogen existence is moderated by several factors within the wetland: air, water and substrate interface. These factors include dissolved oxygen (DO), temperature, concentration of nitrogen, pH, microbial population, types of vegetation and water flow (Bastviken, 2002)

Nitrogen transformation processes include:

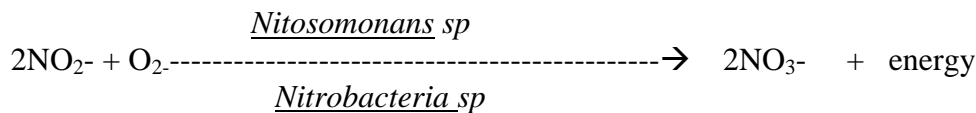
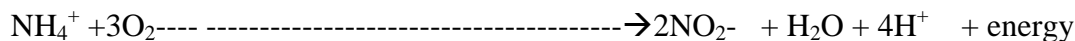
Nitrogen fixation – this is a process whereby bacterial convert dinitrogen gas into ammonium (NH_4^+)

Nitrogen mineralization-this is the conversion of organic nitrogen during the decaying process of organic matter to form ammonium (ammonification) by microbial action. This process could take place in both aerobic and anaerobic conditions. Urea is often formed and is hydrolysed to form ammonia which transforms to ammonium within the water phase



The NH_4^+ can enter different pathway such as assimilation by plants, by microbes to form organic matter or can be immobilized by soil particles.

Nitrification:- NH_4^+ available in aerobic zone in the wetland is oxidized to nitrate by nitrifying bacteria. The first stage is the formation of nitrite, followed by nitrate formation.



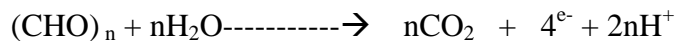
NO_3^- is mobile in the soil solution, thus could be absorbed by plants, leach into ground water and pass via other pathways. The most important pathways for nitrogen reduction and permanent removal are through denitrification (Gosselink and Mitsch, 1993).

Nitrogen assimilation. - This is the transformation of inorganic nitrogen into organic form in the tissue of plants or micro organisms. In the wetlands emergent plants uptake of nutrients

have influence on the seasonal nitrogen dynamics, but the decay of the plants is a potential for nitrogen to return in the form of organic matter and giving rise to ammonium release (Howard, 1995). Organic materials in the sediments, traps nitrogen in organic form, which can be released when there is disturbance of the sediment. During deposition NH_4^+ can also combine with negatively charged soil particles and become immobile depending on the chemical condition of the water.

Volatilization of ammonium. - This is the physiochemical process whereby ammonium is transformed into the gaseous form, ammonia, which may be released to the atmosphere due to the differences in the relative concentration of ammonia between air and water. The wind velocity, solar radiation and vegetation are also said to influence this process (Bastviken, 2002).

Denitrification- This is the microbial reduction of nitrate to N_2 (dinitrogen gas). It can also be referred to as dissimilatory nitrate removal, due to the fact that it occurs in along side with decomposition or oxidation of organic matter. In the presence of oxygen organic materials in the wetlands are readily mineralized by micro organisms



In anoxic situations, micro-organisms utilizes nitrate, Fe^{3+} , sulphate and the least carbon dioxide for respiration depending on the redox potentials (Gosselink and Mitsch, 1993)

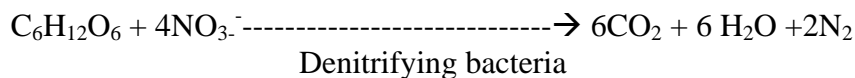
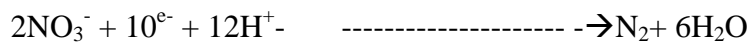
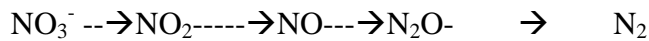
In oxygen limiting situations, i.e. absence of free oxygen gas nitrate is the first compound to accept electrons from an electron donor. The process requires denitrifying bacteria, which are active in an anoxic condition, nitrate rich environment, organic matter presence, pH of 6 – 8.5 and appropriate enzymes (Knowles, 1982).

In wetlands there is usually a vertical stratification with depth showing differences in DO (dissolved oxygen) level, organic matter or suspended solids, and other physiochemical parameters, such as pH and temperature and the redox potentials.

When nitrate becomes limiting the anaerobic bacterial will utilize Mn^{4+} or Fe^{3+} followed by sulphate and lastly carbon dioxide as an electron acceptor in the most anaerobic zone (Kadlec and Knight, 1996).

2.4 STEPS IN DENITRIFICATION

Nitrate is first reduced to nitrite and finally to nitrous oxide or dinitrogen where nitrate, nitrite, and nitrous oxide are electron acceptors and sub sequentially utilized (Hallin, 1998):



This process is responsible for the permanent removal of nitrogen from the wetland. Under natural flooded soil conditions, nitrogen becomes limiting due to denitrification (Simpson et al., 1978). The process of nitrogen flux in wetlands involves different microbial interventions, thus the role of micro organisms is so critical in wetlands application either for urban water treatment, industrial effluent and agricultural waste treatment facilities. Denitrification has been identified as the primary reason wetland serves as nitrogen sink (Crumpton et al., 1993). Other nitrogen removal routes such as plants assimilation, microbial assimilation, dissimilatory reduction to ammonium nitrogen may account for 1- 34% of the net nitrogen loss (Stengel et al 1987; Barlett et al 1979).

Wetlands plants are important because they aid aeration in the water column during photosynthesis, the roots also provide surface for the attachments of microbes. The decayed plant materials constitute the organic materials, which build up and in turn affect the characteristics of the wetland sediment. The presence of algae is also a notable characteristic in a wetland. Algae are important for primary productivity and aeration. The type of vegetation in the wetland area could be the basis for characterization of a wetland. Some are described as forest wetland, others mangrove wetland and savannah wetland. On a general note, however, wetlands are recognized by emergent plants, submerged plants and shallow water level with a muddy bottom. The dead plant materials are vital sources of organic matter as energy sources for micro organisms in the sediments (Gosselink and Mitsch 1993).

2.5 SOME RELATED STUDIES

There are past investigations relating to nitrogen flux and denitrification in wetlands situation and these include: Bastviken (2002): Nitrogen removal in treatment wetland model and microcosm studies; Gamble et al, (1977): Denitrifying population in natural soil samples. Other investigations focus on the control conditions for denitrification such as the addition of external energy sources and various impacts on denitrification (Isaac and Hanze 1995; Andersson et al., 1995; Hallin, 1998). Sakadevan et al (1998) reported studies on the impact of cadmium, copper and zinc on denitrification in surface wetland sediments.

2.6 RESEARCH QUESTION

The research question is whether there is any impact of heavy metals on the activities of denitrifying bacteria in the wetland sediments.

2.7 RESEARCH OBJECTIVES

The objectives of the research include:

1. To ascertain if there was denitrification in the studied sediments
- 2 .To find out the possible impact of some concentrations of heavy metals on the rate of denitrification.
3. To compare the rate of denitrification in the different metal treatments and the zero treatment (the control).

2.8 STATEMENT OF HYPOTHESIS

Null hypothesis (Ho): Heavy metal such as Pb, Zn, or Cu does not have any significant effects on the rate of denitrification independent on the concentration

Alternative hypothesis (Hi): Heavy metal such as Pb, Zn, or Cu has significant effects on the rate of denitrification in a certain concentration range.

CHAPTER THREE

3.0 METHODOLOGY

3.1 SITE FOR SEDIMENT SAMPLING AND JUSTIFICATION

Sediment was taken from the wastewater treatment plant wetland at Nykvarn, Linköpings Sweden (58°26'N and 15°36'E). The wetland was established in 1993 to serve as a secondary treatment plant for the municipal wastewater nutrients removal. It consists of six ponds A1B1, A2B2, A3B3, A3B3 arranged in series with an average area of 1000m² and depth of 10-30cm. The wastewater at stage A3B3 is expected to have attained high level of purification before the water is channelled to the city river Stångån.

The common flora in the ponds includes: *Typha latifolia*, *Lemna minor*, *Phalaris arundinacea*, *Elodea canadensis*, *Glyceria maxima* and *Spirogyra sp.* By design this constructed wetland is expected to remove nutrients from the flux from the wastewater from the sewage from the city. As the water moves from one pond to the other the aquatic plants, along with biochemical processes taking place in the system help to reduce the level of nutrients in the water. Prominent among the nitrogen removal pathway is denitrification. A wastewater like this has the potential for accumulating loads of heavy metals, such as lead, copper, zinc and others, thus it is justified to examine the possible impact of these heavy metals on the efficiency of biochemical process such as denitrification.

The field studies were carried out between august 2005 and march 2006, using samples of sediment from this site in a laboratory experimental setup.



Figure 1 Map showing site sediment was sampled (Linköpings)

MATERIALS AND METHODS

The investigation of the possible impact of heavy metals in wetland sediments on the denitrification activities of micro organisms in a wetland receiving waste water from an urban waste water treatment plant was carried out by laboratory analysis, drawing upon the acetylene inhibition method in sediments slurries.

The laboratory procedure was chosen in order to have control over different environmental factors such as sunlight, varying temperature, pH and plants which will have a combine influence on denitrification. Sediments were taken along with the bacteria typical of wetland sediments. An inherent content of organic matter serving as energy source for the denitrifiers was relied upon. The organic carbon can in the alternative be ensured by external source such as the addition of ethanol, methanol, lipids, and acetate as electron donor. These are important conditions for denitrification process. The substrate from the wetland had been chosen in order for the findings to have relevance to the wetland situation defined by the objective of the study. The sediment was subjected to incubation. Nitrate was added at a specific concentration, 1.4mg/l.

To ascertain the production of N_2O compound from denitrification, gas chromatography was used in estimating the amount of gas evolution. The production of N_2O and N_2 is the end products which qualify the process of denitrification; N_2O could be intermediate or final product in some denitrification depending on the method employed for the investigation. Glass bottles were used in the experimental setup.

3.2 MEASURING THE RATE OF DENITRIFICATION

There are some methods useful in the estimation of the rate of denitrification. The approach may be to measure nitrate consumption or nitrite production. This approach, however, does not account for the total breakdown of nitrate (Hallin, 1998). Another approach is to measure the amount of N_2O concentration produced as a result of acetylene inhibition technique. This has been used to investigate denitrification in soil (Pell et al., 1998; Parsons et al., 1991); activated sludge (Hallin, 1998) and sediments (Bonin and Raymond, 1990).

The acetylene inhibition technique essentially results in reversible inhibition of N_2O reductase enzymes by acetylene, preventing the N_2O from further reduction to N_2 . This is important due

to the difficulties in estimating N_2 gas. In this study the standard concentration of 10% acetylene (Yoshinari and Knowles, 1976) was used in the head space of the slurries in the glass bottles. N_2O was determined with a gas chromatograph connected to an xx detector. Acetylene inhibition technique is said to have some weakness such as the possibility of also inhibiting nitrification and the production of N_2 despite the inhibition of N_2O reductase (Seitzinger et al., 1993).

However, most investigations in related studies have relied on this acetylene inhibition technique (Hallin, 1998). The addition of nitrate into the aquatic sample help to avoid these two weaknesses. The system will not rely on nitrifiers to supply nitrate necessary for denitrification efficiency.

An alternative techniques, include a ^{15}N tracer technique (Nishio et al., 1983), where, $^{15}NO_3^-$ is added to the denitrifying samples and the dilution of the isotope to form ^{14}N Nitrate is measured according to Davidsson et al., (1997). The nitrate conversion to N_2 in this case is analysed by mass spectrometry.

Another technique is the N_2 flux procedure which is the direct measurement of the N_2 produced during denitrification (Seitzinger, 1993). The limitation associated to this includes the risk of contamination from the atmospheric N_2 . Thus, an air tight background will be needed for the measurement (Bastviken, 2002).

The experiment to determine the effects of heavy metals, Cu, Zn and Pb on denitrification process in wetland sediments were carried out in the laboratory under an anaerobic condition, which is an environmental requirement for the denitrification process. A pre-experiment was carried out with various controls, to determine the impact of the absence of nitrate and or acetylene on the results of the denitrification assay.

3.3 PREPARATIONS

A buffer solution was made: phosphate buffer 15ml of KH_2PO_4 (0.2M), W1 and 15ml of $NaHPO_4$ (0.2M), W2 made into 800cc of distilled water filled up to 900cc and shaken

thoroughly. 45ml of the buffer solution was administered to 10, 123ml bottles (5 treatments and 2 parallel) needed for the experiment and then sealed with butyl rubber septa. The bottles with the buffer solution were subjected to gas exchange operation with the nitrogen gas by depressuring and pressuring the bottles at least five times. Afterwards and the bottles were autoclaved for 20minutes at 120°C

Nitrate salt solution was prepared and gas exchange was performed as above. Acetate solution was also prepared (Sodium acetate). 1.4g of KNO₃ was dissolved in 100ml of distilled water and 0.57g of acetate in 100ml of distilled water.

Sediment samples were taken from the wetland described above. The sediment materials were filtered, homogenized and left to settle overnight. Thereafter the water above the sediment was sucked out using a tube and syringe. 5ml of this sediment were given to each of the parallel bottles set A/B for the various treatments 1,2,3,4 and 5 (See Table1). The dry weight of the sediment was determined by measuring about 15ml of the slurry placed in a foil plate of known weight and dried at 105°C overnight. It was allowed to cool and was kept dried in a desiccator to ascertain the new weight and determine the proportionate dried weight of the sediment that was used in the experiment.

Table: 1 Pre experiment to determine the implication of the various control treatments on denitrification.

T	A	B	CHCOONa	NO ₃ ⁻	C ₂ H ₂	Sediment
1				x		x
2					x	x
3			x			x
4			x	x	x	x
						x
5						

(NB: x means application)

Treatment Levels (for concentrations see appendix 4b)

45ml of buffer to stabilize the pH necessary for the anaerobic environment.

2.5ml of nutrient

1ml of nitrate,

1ml of acetate,

5ml of sediment,

10% volume of gas phase, C_2H_2 making up to 7ml.

These sets of control experiment were then placed in the climate room maintained at 20° C and on a shaker platform and subsequently gas samples were taken at different time intervals; which were 1hr , 2hrs, 4hrs, 8hrs, 24hrs and 48hrs which corresponds to T0, T1, T2, T3, T4 and T5 respectively. 1ml gas sample was taken from each assay bottle and diluted in a closed 15ml bottle from which 0.3ml of it was introduced into the GC for analysis. The obtained area marked by the GC reader corresponds directly to the concentration of N_2O . There were responses to the various parameters under consideration (Sets of control)

Setup 1. The system had good source of nitrate, nutrient sediment and acetylene. The result shows a progressive rise in the level of N_2O that was produced during denitrification and that the organic matter in the sediment can sustain a steady denitrification for the incubation time used.

Setup 2. There was no nitrate and acetate but acetylene. There was lower N_2O production at the initial time, but it became more towards the last hours of the experimental sampling .The conclusion is that some nitrate and electron donors were present in the sediment.

Setup 3. This was a control for acetylene inhibition. It was observed that the system could only estimate a small proportion of N_2O as the N_2O may have likely undergo further transformation, which means that the method worked under the conditions planned for the assay.

Setup 4. Had all the materials, nutrient, nitrate, acetate, acetylene and sediment. There was a steady increase in the gas concentration with time

Experimental setup 5 without nitrate, acetate, and acetylene, shows a negative value or no response at the beginning and as the time progresses towards the end there was some release of N_2O , which can be linked to the nutrient in the sediment

Conclusively, acetylene inhibition method worked within the framework setup for the experiment.

3.4 MAIN EXPERIMENTAL SETUP

The materials include: wetland sediment, nitrate, nutrient, buffer and acetylene as noted above, with chlorides of Cu, Zn and Pb.

Method: Wetland sediment was taken from the wetland site in Linköpings Nykvarn wastewater treatment wetland. The sediment was filtered (1mm sieve), homogenized and allowed to settle. The water level was reduced there after.

15ml of the sediment was taken and placed in the dryer maintained at 105°C for 24hrs and the dried weight of the sediment was obtained. 45ml of the buffer were introduced to the 123ml bottles sets of each of the experimental setup. 5ml of the wet sediment samples (inoculums) were introduced. Nitrate (1ml), nutrient solution (trace of mineral salts 2.5ml) and acetylene (7ml) 10% of the gas phase were also added.

Calculated amount of the metals: Cu, Zn and Pb (see appendix 4) were made in 25ml of distilled water so that 0.1, 0.25, 0.5 and 1ml represented 100, 250, 500 and 1000 mg/kg respectively, and as used in treatments 1, 2, 3 and 4 respectively. A control without any metal treatment was included as the zero treatment 0T, for each of the set ups, there was three parallels A, B and C. The experimental sets up in each case were placed on a rotary shaker for the duration of the experiment. First installed in the climate room during the experiment 1a (Initial Cu trial) and 1b (Initial Zn and Pb trials), and in the room temperature maintained in the laboratory (20°C) for experiment 2(back up trials for Cu, Zn and Pb).

Table: 2 treatment description

Treatment levels mg/kg/dry wgt.		Cu 1 Treatment	Zn 2 Treatment	Pb 3 Treatment
T0	0	1T0	2T0	3T0
T1	100	1T1	2T1	3T1
T2	250	1T2	2T2	3T2
T3	500	1T3	2T3	3T3
T4	1000	1T4	2T4	3T4

N/B The treatment were designed in three replicates

3.5 SAMPLING AND DATA

Each experimental setup gas samples were taken after 1hour, 2hrs, 4hrs, 8hrs and 24 hrs. In each case 1ml of the gas phase was taken with a syringe and injected into 15ml bottles closed with rubber stoppers.

A total of 15 samples were withdrawn for each of the five sampling occasions, generating a total of 75 (5x15) sampling data in the 1st experimental procedure ; and making a total of 3x 75 that is 225 samplings for all the three trials.14 samples were taken in the experimental setup 2 with six sampling period; generating 84 (6x14) sampling data.

3.6 DATA COLLECTION

The acetylene inhibition technique blocks the formation of N₂ gas and as such N₂O gas is the main product formed during the denitrification process. To estimate the amount of N₂O concentration in each sample a gas chromatograph equipped with an electron capture detector GC/ECD was used and with a computer system and GC packed software to show the various detected peaks of gases(as described previously).

First we calibrated with known concentrations of pure N₂O and registered the obtained peak areas. To calibrate, we introduced 2ml of pure N₂O into a 123ml bottle which was the source for dilutions: i.e. 1ml, 0.5ml, 0.25ml, 0.1ml was withdrawn and injected into four other 123ml bottles respectively. 0.3ml from these 4 bottles was injected into the GC and the peaks

areas were observed. Higher concentration in the samples, which cannot be processed by the GC/ECD, will require further dilution before re injection. The analysis were performed for the various samples using 0.3ml of the diluted gas, injecting it into the GC and noting and registering the peak for N₂O.

There were occasions for dilution for the last two sampling occasions for the experimental set up. 0.5ml of the samples was withdrawn from 15ml bottles, injected into 12.3ml bottles and mixed, and then from these, 0.3ml was injected in the GC. Peaks were observed and registered. These values form the basic data, which were primary research data. The registered peaks were then subjected to further deductions to convert the area to concentration.

3.7 DATA ANALYSIS AND STATISTICS

The research aim was to find out if there was any impact of the treatment (heavy metal) on the rate of denitrification. One can estimate the rate of amount of N₂O evolved from the gas phase of the experimental setup at the various time intervals. We can ascertain this amount of N₂O produced to be a dependent (y) variable, while time an independent (x) variable. The amounts of N₂O produced by the parallel were taken as data(y). A standard curve was obtained from the area results of the calibration data and y (N₂O concentration) was estimated from the standard curve formula and the concentration was then used for a linear regression.

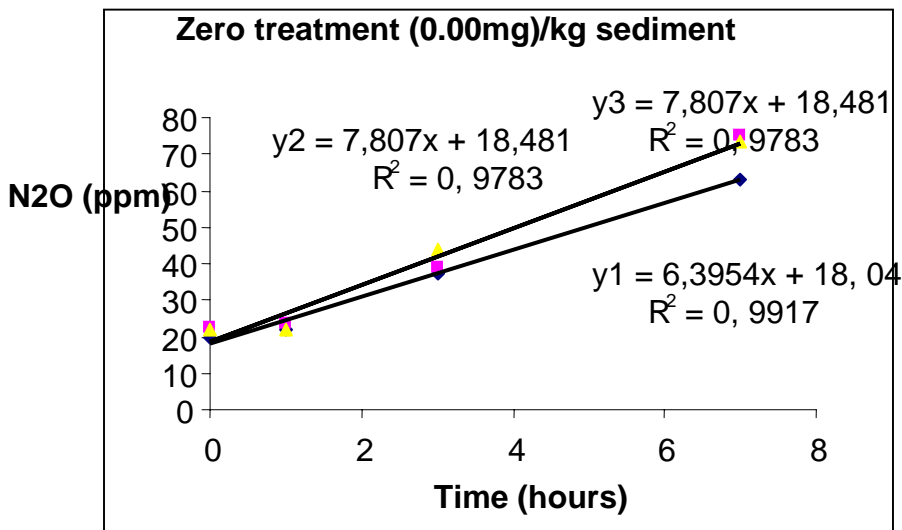
The coefficient of correlation (R) was estimated from the R Square (fitness of the curve) and comparison was made between the control, and the various metal treatments and their concentration levels. The slope of the graph represents the rate of denitrification, thus for each of the treatment these rates were compared with that of the control. The slope of the graph is the change in Y/change in X. The statistical tools used were Excel for the linear regression and the graphs and SPSS for multiple regression analysis and a confidence interval of 95%. Concerning the R² value, high values indicate a positive trends or fitness of data while low values indicate poor fitness of data.

CHAPTER FOUR

DATA PRESENTATION, ANALYSIS, RESULTS AND DISCUSSION

The first trials on Cu were made a day after the sediment samples were collected from Nykvarn. There was a substantial denitrification occurring (appendix 1 table 1). The trials for Zn and Pb could not follow as scheduled, but were carried out after two weeks. The results showed poor microbial activities compare to those for copper and the activity had ceased after eight hour of incubation. The possible explanation to these observations will be given in the discussion. This situation necessitated the back up trials, which was the experimental setup 2 for treatment levels T3 (500 mg/kg) and T4 (1000 mg/kg). See Table 2. Data are presented in Appendix 1(tables 6, 7, and 9).

The results of experimental set up 1a, where Cu was used in treatment at 4 levels, are described in the following graphs and the details of data appendix (1).



Figures 2 Copper control treatment 000mg/kg of sediment (Cu 1T0) with y1, (y2, and y3 same regression line) representing A, B, C parallels respectively.

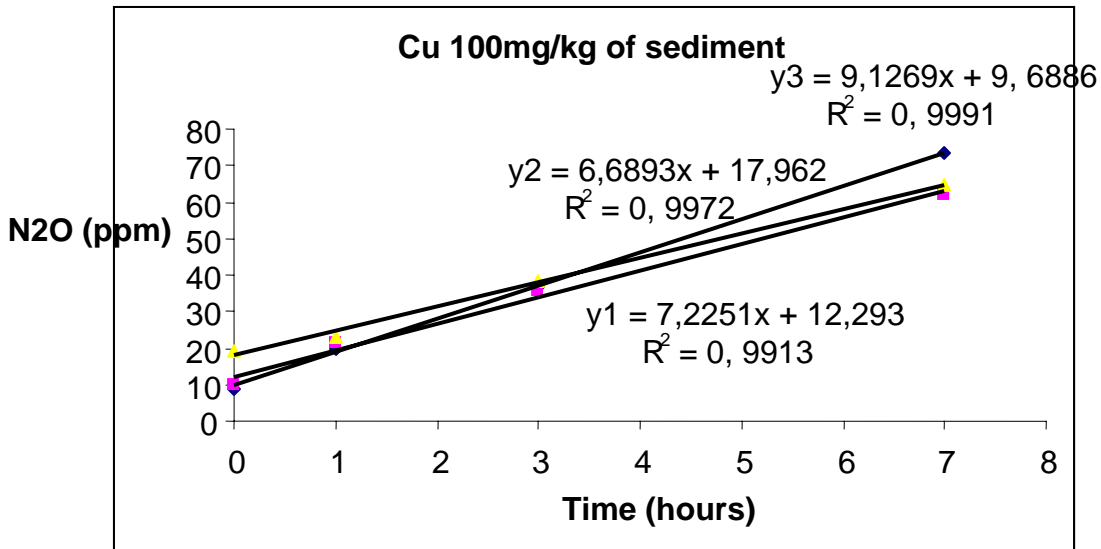


Figure 3 Cu treatment Experiment 1a (1T1) 100 mg/kg sediment treatment, with y1, y2, y3 representing parallels A, B, C respectively.

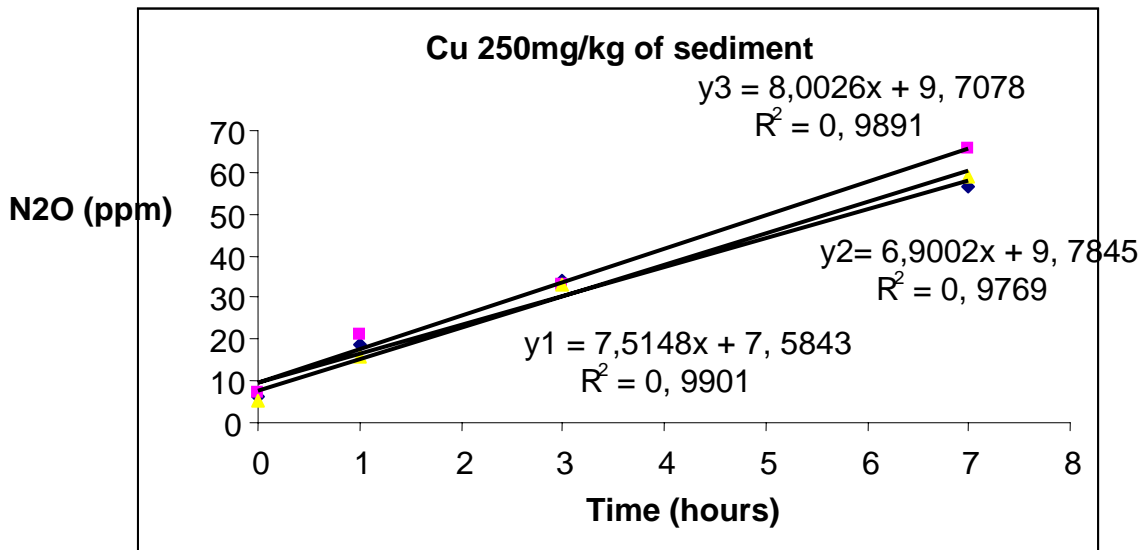


Figure 4 Exp 1a Cu (1T2) 250 mg/kg sediment treatment, with y1, y2, y3 representing parallels A, B, C respectively.

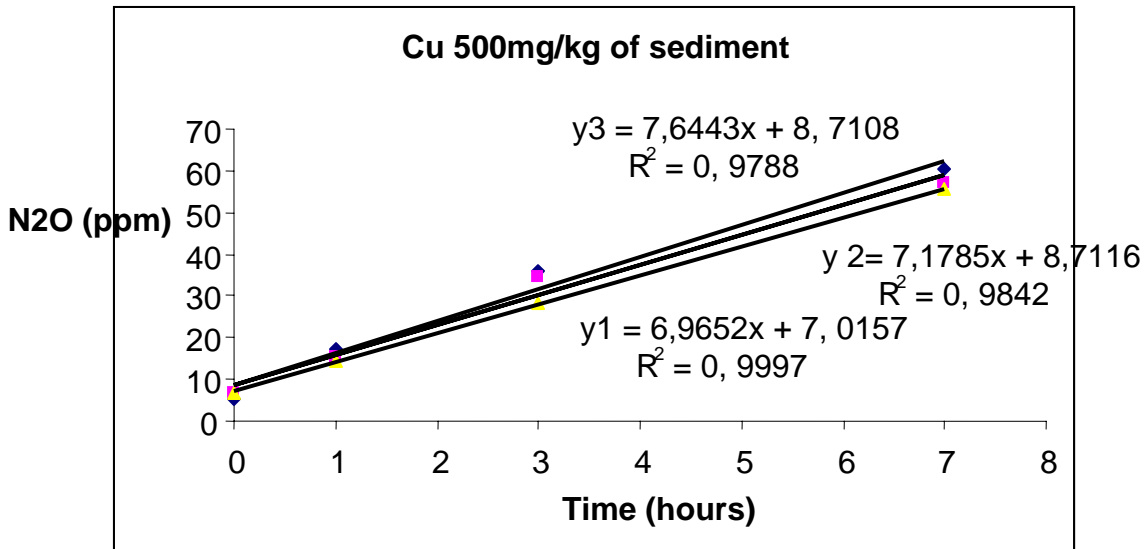


Figure 5 Exp 1a Cu treatment (1T3) 500 mg/kg sediment treatment, with y1, y2, y3 representing parallels A, B, C respectively.

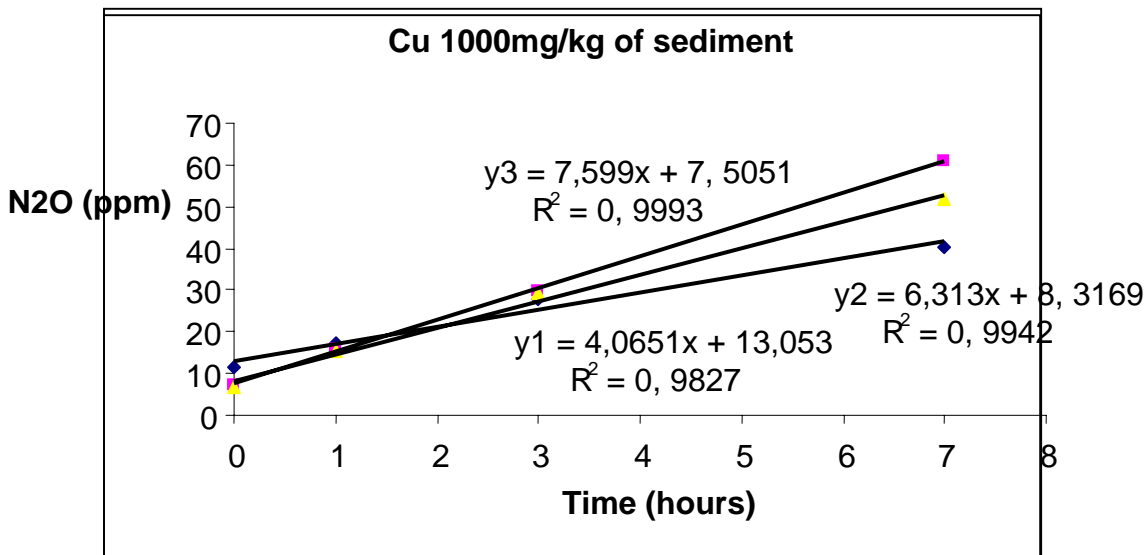


Figure 6 Cu trials 1T4 1000 mg/kg sediment treatment, with y1, y2, y3 representing A, B, C parallels respectively.

The summary of results from the first trial with Cu is shown in table 3. The rate of N₂O production as an evidence of denitrification by the denitrifying microbial community in Cu treatment is given. This, however, cannot be asserted conclusively until we are able to compare these values with other treatments. It is shown here, however, that the rate for the control experiment (that does not have any copper salt) to be between 6.4 ppm/hr and 7.8 ppm/hr, which the treatment 1T1 also seems to give. It means at 100 mg/kg treatment level

there seems to be no effects of Cu on denitrification. There were some stability and little reduction of rate in subsequent levels; these are summarized in the following table (Table 3).

In a stepwise evaluation of the rate dynamics of the Cu treatment we can observe that there were no effects in the 100 mg/kg trial. This result relates to findings reported by Sakadevan et al (1999); that there were no effects of Cu at a treatment level of 100 mg/kg of sediment on denitrification. At the Cu level 250mg/kg, the rate was 7.5 ppm/hr compare to the control 7.3, seems no much difference. At 500mg/kg sediment treatment, the rate of N₂O production was 7.0ppm/hr; this shows some drop in the N₂O production rate. Finally, at 1000mg there was a mixed response, i.e. a tendency for further inhibition or for tolerance. Among the parallels in the 1000mg Cu treatment we recorded a range of 4-7.5ppm/hr. Sakadevan et al (1999) reported that Cu treatment at 1000mg /kg shows less inhibition, in effect, there could be a microbial tolerance, which may seems to produce a recovery rate in some samples.

Table 3 Cu treatment Exp 1a summary of denitrification rate

Cu treatment mg/kg of sediment	Production rate of N ₂ O (slope) ppm/h	R	R ²	Remarks on rate ppm/h
000.0	7.3	0.98	0.98	6.4 -7.8
100.0	7.6	0.98	0.98	6.5 - 9
250.0	7.5	0.98	0.97	7 - 8
500.0	7.0	0.98	0.98	7 - 7.5
1000.0	6.0	0.99	0.99	4.0 – 7.5

Experiment 1b Trials of Zn and Pb

The experimental set up 1b was carried out two weeks after field sampling of the sediment. It was not a specific objective of our investigation, but, a result of some constraints there were some delay in the schedule. The results of the initial experimental trial of Zn and Pb are shown in table 4 below and in figures 4.6 a-j in the appendix 3.

Table 4 Zn and Pb treatment results of initial experiment 1b (Detailed results in Appendix1 Table7)

Treatment	Production rate N ₂ O ppm/h	R2	Remarks on rate
2T0A	<0.1		
B	<0.1		
C	<0.1		
2T1A Zn	0.60	0.92	0.56
B	0.56	0.90	
C	0.51	0.93	
2T3A	0.76	0.94	0.69
B	0.6	0.92	
C			
2T4B	0.33	0.91	0.44
C	0.55	0.98	
3T0A	0.24	0.79	0.36
B	0.35	0.94	
C	0.48	0.95	
3T1A Pb	0.29	0.98	0.24
B	0.22	0.99	
C	0.19	0.96	
3T2A	0.39	0.96	0.45 (0.36)
B	0.34	1.00	
C	0.61	0.97	
3T3A	0.25	0.89	0.33 (0.52)
B	0.45	0.96	
C	0.28	0.86	
3T4A	0.18	0.79	0.42 (0.53)
B	0.57	0.96	
C	0.52	0.99	

NB. The rate for 3T3A was below: 0.5 and for 3T3C: 0.6 (Pb treatment 500 mg/kg sediment)
3T4A, from zero to one hour the rate was 0.5 ppm/h, but decreases rapidly until 3h, also with the parallels.

From this experiment it is shown that the initially relatively high rates of nitrous oxide accumulation decreased after 4 hours incubation, from there, they were at zero. In order to see if there were any effects of the supplied metal concentration levels, I looked into the first four

measuring points by letting the 1-hour sampling be set to zero and the others adjusted accordingly, this was taken as there could be a natural delay in microbial activity in a system.

The R^2 values (0.8-1.0) show that there is an almost linear relationship for most of the accumulation occurring during the first four hours. The graphs then seem flattens out at some point before ten hour. There was also a remarkable difference between the replicates, which seems very unusual as the experiment was conducted in the same condition (Appendix 3 (Figures 7 c, d, e, f, g, and i)).

EXPERIMENTAL SETUP 2 TREATMENT OF Cu, Zn and Pb

In view of the quality of results from the initial Zn and Pb trials we decided to have a re- trial of all the three metal under investigation: Cu, Zn and Pb with a fresh sediment, using two levels of treatment 500mg/kg and 1000mg/kg (T3 and T4 respectively).

The results of the second trials (Experimental setup 2) came out as follow

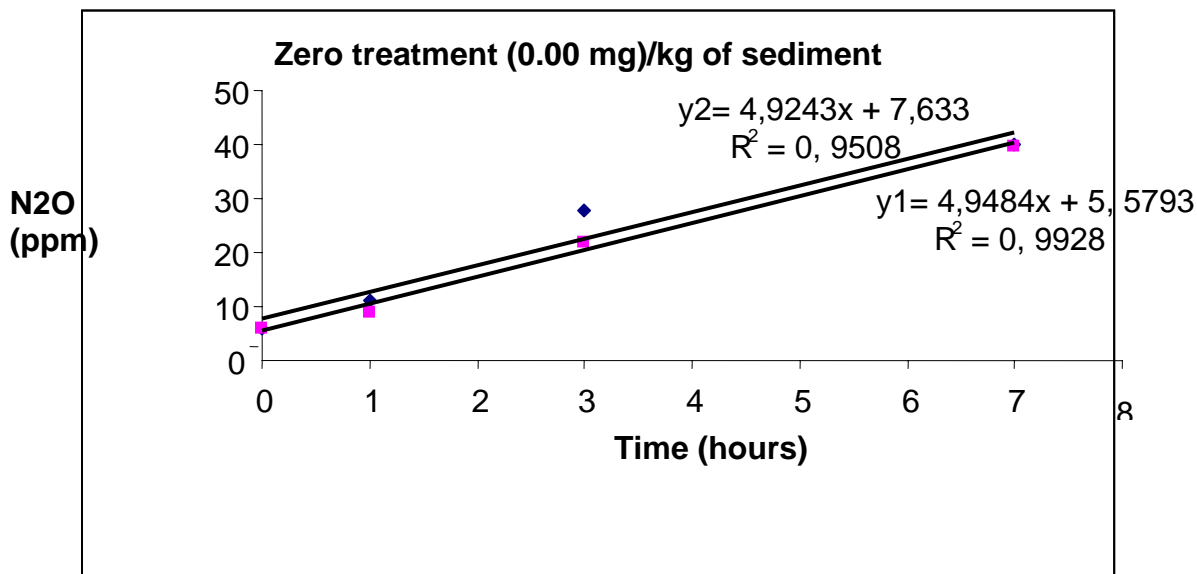


Figure 8 Comparison of the control treatment (0T0) with replicates 1 and 2. The replicate seems to demonstrate close margin.

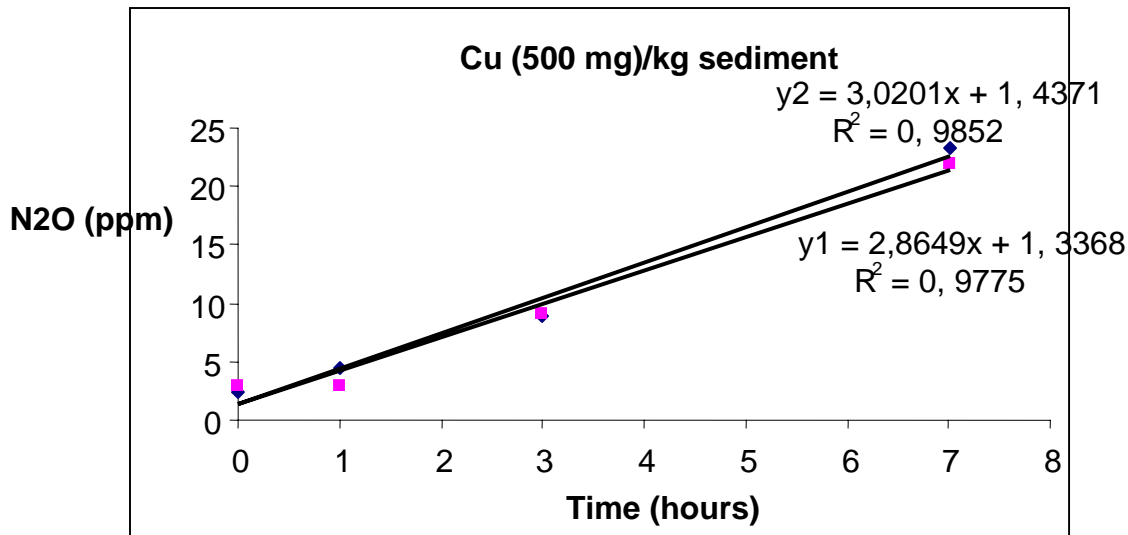


Figure 9a

A

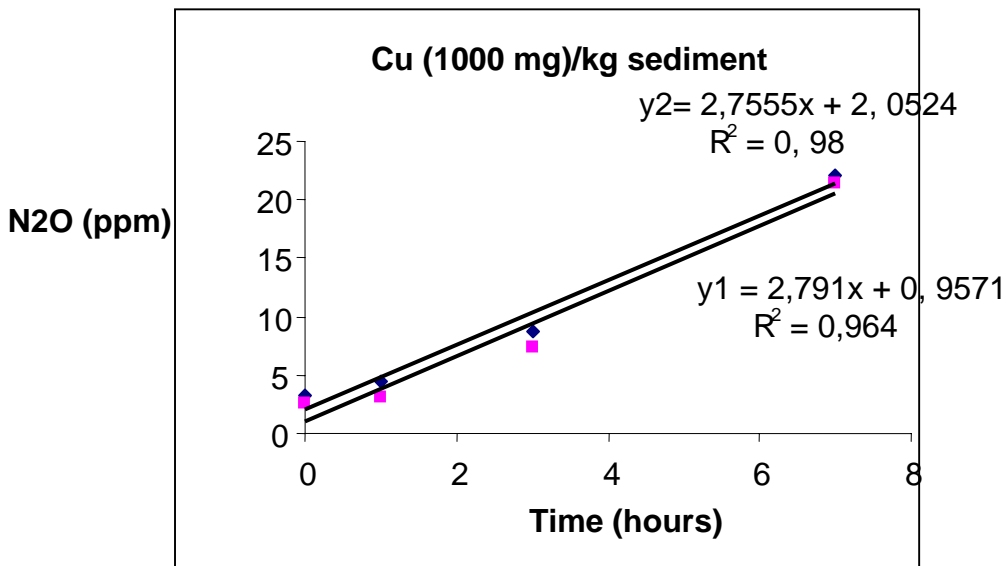
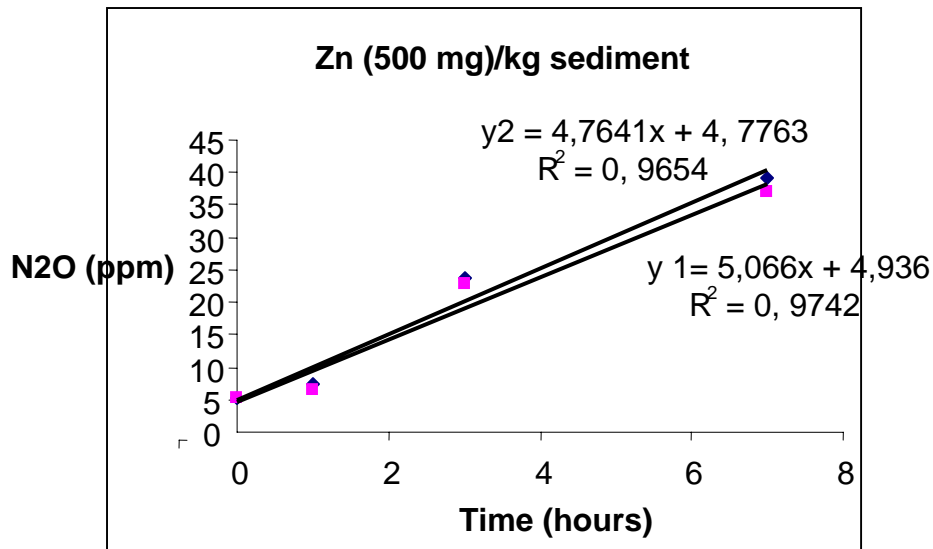
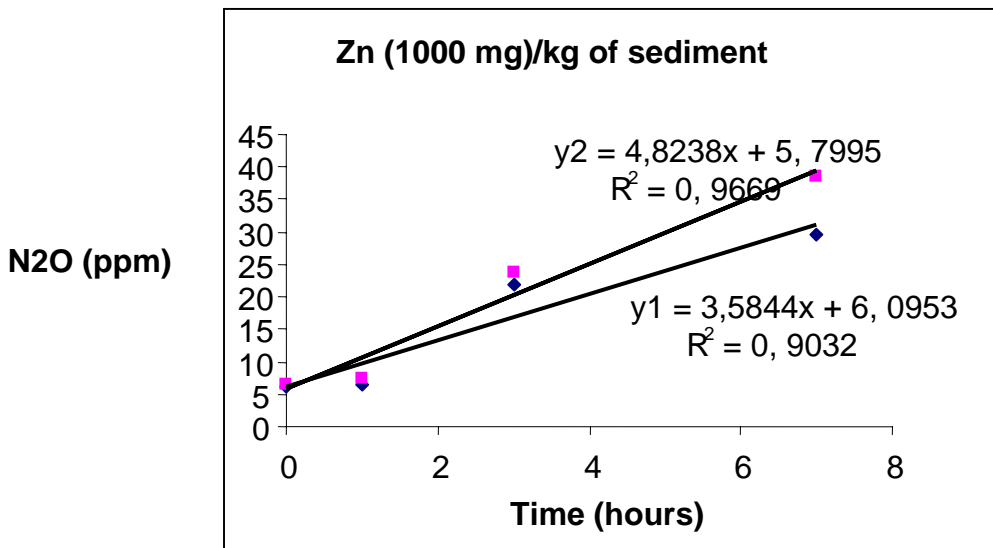


Figure 9b

Figures 9A/B: Cu treatment (Figure 9A) with replicates of 1T3 (500mg/kg), while figure 9B, Cu treatment with replicates of 1T4 (1000 mg/kg).



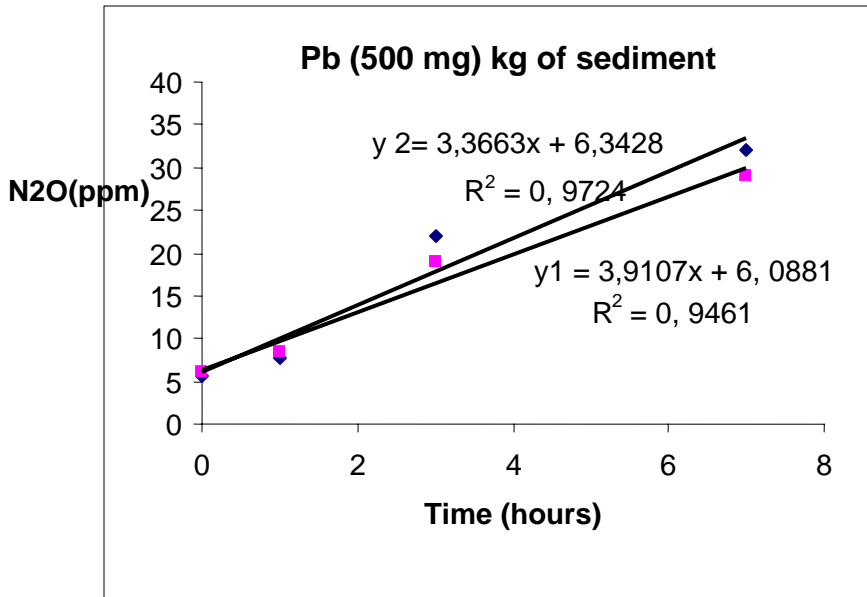
A



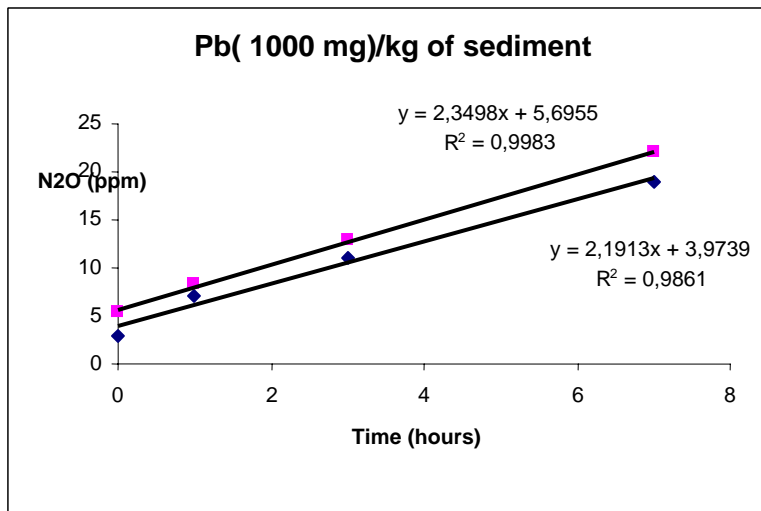
B

Figures 10A / B

Zn treatment (Figure 10A) with replicates of 2T3 (500 mg/kg), while Figures 10 B with replicates of 2T4 (1000 mg/kg).



A



B

Figures 11 A / B: Pb treatment (Figure 11A with replicates of 3T3 (500 mg/kg), and figure 11 B Pb treatment with replicates of 3T4 (1000 mg/kg).

TABLE 5 SUMMARY TABLE OF EXPERIMENTAL SET UP 2.

Trial	metals mg/kg	R^2	R	R^2	R	Slope a y1	Slope a Y2	Remarks on rate ppm/h
0T0	Zero 0	0.95	0.95	0.99	0.99	5.0	5.0	5
1T3	Cu 500	0.98	0.99	0.93	0.96	3.0	3.0	3
1T4	Cu 1000	0.95	0.97	0.99	0.99	2.7	2.8	2.75
2T3	Zn 500	0.98	0.99	0.94	0.97	4.7	5.0	4.85
2T4	Zn 1000	0.96	0.98	0.95	0.97	4.8	3.6	4.2
3T3	Pb 500	0.91	0.95	0.99	0.99	3.4	3.9	3.65
3T4	Pb 1000	0.92	0.96	0.95	0.97	2.3	2.2	2.25

The above results in table 5 were obtained from the second experiment (experiment 2). The initial Zn and Pb treatment (Experiment 1b) showed no practical response from the sediment and the various treatments (Table 4.2 appendixes 1). The reasons could be the time laps before the experiment was performed and the time when the sediment was sampled from the field. While the first treatment was conducted for Cu one day after the sampling, the Zn and Pb treatments were carried out after two weeks. Microbial processes taking place in the sediment may have resulted to the loss of the energy source before the experiment was performed in Exp 1b. Other explanation could be that there was a loss of denitrifying bacteria or the bacteria may have been in activity when the sediment was fresh and may have used up the energy source.

Results from the experimental setup 2 shows a slight tendency towards inhibition for Cu as compare to zero treatment; denitrification rate was about 3ppm/h for Cu 500 mg/kg while the controls was 5 ppm/h; there was a stability of this rate for the Cu even at 1000 mg/kg treatment level, which may also indicate some level of tolerance of the bacterial at that Cu

treatment level (table 4). Zn shows comparable denitrification rate to the control at 500mg/kg sediment treatment (4.85ppm/h) and a lesser rate of 4.2ppm/h at 1000 mg/kg of sediment treatment. Pb shows a denitrification rate of about 3.7 ppm/h at 500 mg /kg of sediment treatment and also. 2.25 ppm/hr in1000 mg /kg of sediment, such lower rate for Pb were expected due to high toxicity of Pb to micro organisms (Sakadevan et al 1999). The data were further analysed using multiple regression to see if there is any significant response by the three metals: Cu, Zn and Pb, the results are presented as follows (Table 6a/b)

Table 6
Regression (a)

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	10,474	1,312		7,982	,000
	VAR0000	4,191	,342	,972	12,267	,000
	2					
	VAR0000	-1,343	,483	-, 208	-2,780	,007
	3					
	VAR0000	,378	,483	,059	,783	,437
	4					
	VAR0000	-1,261	,483	-, 195	-2,609	,012
	5					
	VAR0000	-9,180	1,856	-, 344	-4,947	,000
	6					
	VAR0000	-4,915	1,856	-, 184	-2,649	,010
	7					
	VAR0000	-4,533	1,856	-, 170	-2,443	,018
	8					

a Dependent Variable: VAR00001

Model Summary (b)

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	,955(a)	,912	,901	3,66393

a Predictors: (Constant), VAR00008, VAR00002, VAR00007, VAR00003, VAR00004, VAR00006, VAR00005

The multiple regression models formular:

$$Y = b_0 + b_1*t + b_2*t + b_3*t + b_4*t + b_5*t + b_6*t + b_7*t$$

The b's are the regression coefficients, representing the amount the dependent variable y changes when the corresponding independent changes 1 unit. The b0 is the constant, where the regression line intercepts the y axis, representing the amount the dependent y will be when all the independent variables are 0.

Where, b_0 is the B constant, $b_1, b_2, b_3, b_4, b_5, b_6$ and b_7 are the beta for the various variables 2-8 respectively.

Cu is b_5 , Zn b_6 and Pb b_7 .

$$Y_0 = 10.47 + 4.19 \cdot 0 + -1.3 \cdot 0 + 0.34 \cdot 0 + -1.2 \cdot 0 + -9.1 \cdot 0 + -4.9 \cdot 0 + -4.5 \cdot 0$$

$$Y_0 = 10.5$$

Y_1 intercept for Cu:

$$Y_1 = b_0 + 4.19 \cdot 0 + -1.3 \cdot 0 + 0.34 \cdot 0 + -1.2 \cdot 0 + -9.18 \cdot 1 + -4.9 \cdot 0 + -4.5 \cdot 0$$

$$Y_1 = 10.47 - 9.18 = 1.3$$

Y_2 , intercept for Zn:

$$Y_2 = b_0 + 4.19 \cdot 0 + -1.3 \cdot 0 + 0.34 \cdot 0 + -1.2 \cdot 0 + -9.18 \cdot 0 + -4.9 \cdot 1$$

$$Y_2 = 10.47 - 4.9 = 5.56$$

Y_3 , intercept for Pb

$$Y_3 = b_0 + 4.19 \cdot 0 + -1.3 \cdot 0 + 0.34 \cdot 0 + -1.2 \cdot 0 + -9.1 \cdot 0 + -4.9 \cdot 0 + -4.5 \cdot 1$$

$$Y_3 = 10.47 - 4.5 = 5.9$$

COMPARING RESULTS OF EXPERIMENTS 1 AND 2

A quick summary of the two experiments, the first experiment gives generally; twice as high rate of N_2O production as in the 2nd experiment. More precisely, in the control experiment N_2O production rate was 9ppm/h (Experimental set up 1) , while that of experimental set up 2(1T0) was 5ppm/h. 1T0 in these two experiments it shows a tendency towards less N_2O production in the experiment 2. In these two experiments, the sediment samples were taken from the same pond; samples were homogenized and used a day after field sampling in each case. The difference could be due to differences in the supply of energy from the organic matter for the denitrification, as a result of differences in the dry weight of the sediment or other nutritional difference between the two sediment samples. 1T3 treatment for Exp 1a was 7.2ppm/h while in Exp 2 was 3ppm/h. 1T4 treatment shows a similar trend, it was between 4.0 -7.5ppm/h in Exp 1 while in Exp 2 we observed approximately 3ppm/h.

The results in Exp 1b show some poor response for N_2O production, when the sediment was used after two weeks. It was indicated that sediment should be used when it is fresh so as to

avoid drying, water accumulation, freezing, loss of energy and value and microbial community and enzyme activity resulting from prolong storage (Andersen 1987, Dick et al 1996, and Öhlinger 1995). Nevertheless, we used our sediment within a day, thus, the differences in the result would rather be a product of the dynamics in the wetland influent vis-avis the microbial activities and other factors outlined earlier in the discussion. Sun et al (1999) and Dunne and Culleton (2004) indicated the possibility for differences in the results between trials in the same wetland land sediments due to numerous interacting factors that define the water quality and the sediment at different time.

CHAPTER FIVE

SUMMARY AND CONCLUSION

The research aimed at finding out if denitrification occurred in the wetland sediment and if there were any effects of heavy metals (Cu, Zn and Pb) at different levels of concentration on the rate of denitrification process. For this purpose we compared the rate of denitrification resulting from the various treatments. The null hypothesis was: there were no effects of Cu, Zn and Pb on the rate of denitrification and an alternative hypothesis, which stated that the different concentrations of heavy metal Cu, Zn and Pb have significant effects on the rate of denitrification.

In the acetylene inhibition technique, N_2O gas is a major indicator of denitrification as N_2 as the final product is restricted by the C_2H_2 introduced into the gas phase of our bottles (Hallin 1998). Certainly, from our studies we observed that N_2O gas was detected from the gas samples injected into the GC

What were the effects of the heavy metals on the rate of denitrification?

Denitrification is an important process in nitrogen cycle. In a wetland situation it account for a major pathway for nitrogen removal, thus, minimizing the problems associated to high nutrient load such as eutrophication. Heavy metals, like Cu, Zn and Pb, are associated with many materials, which can easily leach into the aquatic system. Thus, it is vital to ask if there is any impacts of heavy metals on this, vital ecological process.

The observed production of N_2O from the experiments, where wetland sediment was treated with different concentrations of Cu, Zn or Pb, were compared with the control (where there was no addition of metal), with a multiple regression the results were presented in table 6a/b, details are in appendix 2.

As a summary, we found that there was no significant effect on the rate of denitrification by Cu at 100 mg/kg concentration. Inhibition of denitrification occurred at the concentration levels: of 500 mg/kg and 1000 mg /kg of sediment. However, Cu at the higher concentration does not seem to create higher inhibition, as compare to 500mg/kg. There seems to be tolerance in microbial response.

The results from Zn trials shows also inhibition of denitrification (Table 5), at 1000 mg /kg sediment the rate of denitrification was reduced from 5ppm/h to about 3.6ppm/h this result however buttresses the fact that higher concentration of Zn may increase the inhibition on the rate of denitrification.

In a similar evaluation between the control samples and treatment with lead (Pb), at 500 mg /kg sediment treatment, there was a reduction in the rate of denitrification 3.6ppm/h as compare to 5ppm/h of the control. Also, slow down in the rate of denitrification was observed in 1000mg/kg sediment treatment; the observed rate of denitrification was 2.25ppm/h (Table 5)

CONCLUSION

In the studies we found out that heavy metals like Cu, Zn and Pb do inhibit the rate of denitrification in wetland sediment when available in high concentration of 500mg/kg of sediment. At lower concentration (≤ 100 mg/kg of sediment) there were no significant impacts on denitrification.

Also, the different heavy metals had different inhibition impacts on the rate of denitrification and inhibition could result from different factors such as the quality of the sediment define by the time after sampling.

SUGGESTIONS

Studies of wetlands sediment is an interesting area useful in environmental management. To be effective in undertaking such a research there is a need for interest and a sound knowledge of the background. Wetland related research have been very interesting to me so, there was a high motivation in all aspects of the studies. As noted in some parts of the work, wetlands studies are relevant in getting research data concerning the human impacts on the ecosystem within a catchments basin.

Granted, the duration of the studies was short, more research questions could be examined in further investigations such as: What happens in the water phase e.g how does the concentration of ammonium changes during denitrification?; What is the rate of denitrification measured during a longer time study, than the ones presented in this study; How does temperature affects the rate of denitrification?; What could be the result if we based our treatment dosage on wet weight, instead of dried weight?

These questions are interesting because in the first place, ammonium is lethal to fish at high concentrations. Measuring the rate of denitrification in a longer period of time will help to assess what happens over time as the wetland continuously receives the heavy metals pollutants. Temperature is equally a vital measure of water quality and process reactions. In the wetland there are daily variations and seasonal variations which do have impacts on microbial processes. In most experiments dried weight is often taken for measurement as water constitutes the highest % of the sediment composition. However, in the field, all wetland is inundated most seasons, thus the question of wet weight evaluation for treatment level may sounds reasonable.

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APPENDIXES

1 Tables of detail treatment results and figures

Table 7 Cu treatment experiment 1a

	Time (hours)	Area a	ppm y1	Area b	ppm y2	Area c	ppm y3
1T0A0 ppm y3	0	899	5,779	1843	22,7	1809	22,05
	1	1550	17,13	1883	23,5	1790	21,68
	3	2542	37,45	2600	38,7	2813	43,68
	7	3585	63,06	4016	74,9	3968	73,56
	21	7475	180,8	6955	166	7762	188,9
	23	9589	240,6	10186	257,5	9599	240,9
1T1A0	0	1077	8,83	1136	9,8	1656	19,1
	1	1700	19,95	1770	21,3	1866	23,17
	3	2516	36,87	2462	35,66	2578	38,26
	7	3967	73,5	3541	61,89	3654	64,9
	21	7323	176,51	6166	143,77	6864	163,52
	23	8971	223,15	9076	226,12	9130	227,65
1T2A0	0	908	6,08	968	7,04	853	5,2
	1	1638	18,77	1768	21,26	1489	16
	3	2379	33,84	2343	33,06	2338	32,95
	7	3328	56,35	3676	65,5	3425	58,85
	21	6394	150,22				
	23	8280	203,6				
1T3A0	0	865	5,3	956	6,85	940	6,6
	1	1454	17,39	1450	15,3	1390	14,22
	3	2263	36,06	2404	34,39	2118	28,28
	7	3476	60,18	3364	57,27	3298	55,58
	21						
	23						
1T4A0	0	1249	11,7	966	7,01	955	6,8
	1	1550	17,13	1487	15,97	1444	15,19
	3	2085	27,6	2194	29,87	2156	29,07
	7	2676	40,5	3498	60,76	3142	51,65
	21						
	23						

	a	b	c		y1ppm	y2ppm	y3ppm	Table 8 Zn and Pb treatment detail results
2T1	272	348	218	0	-0,5	0,09	-0,9	
	522	619	436	1	1,5	2,24	0,77	
	693	736	599	3	2,9	3,2	2,08	
	728	754	594	7	3,15	3,37	2,04	
	713	703	619	21	3,03	2,9	2,24	
2T2	282	348	343	0	-4	0,086	0,05	
	584	678	751	1	1,95	2,7	3,35	
	768	916	999	3	3,49	4,78	5,52	
	905	1030	1128	7	4,68	5,8	6,7	
	892	1067	1136	21	4,57	6,13	6,8	
2T3	390	262	363	0	0,4	-0,5	0,2	
	688	526	724	1	2,8	1,49	3,12	
	891	701	1020	3	4,56	2,9	5,7	
	991	713	1124	7	5,45	3,03	6,7	
	1032	749	1143	21	5,82	3,3	6,8	
2T4	1126	816	529	0	6,7	4	1,5	
	908	1114	816	1	4,7	6,6	4	
	923	1162	976	3	4,8	7	5,3	
	925	1312	982	7	4,9	8,4	5,4	
	940	1314	1044	21	5	8,45	6	
3T1	377	211	193	0	0,3	-0,9	-1,08	
	458	298	264	1	0,95	-0,3	-0,55	
	577	397	354	3	1,9	0,5	0,13	
	605	436	417	7	2	0,8	0,6	
	622	398	413	21	2,3	0,5	0,6	
3T2	241	451	524	0	-0,72	0,9	1,5	
	389	520	717	1	0,4	1,44	3	
	538	651	931	3	1,58	2,5	4,9	
	579	710	949	7	1,9	3	5	
	709	687	1164	21	3	2,8	7	
3T3	269	275	226	0	-0,5	-0,5	-0,8	
	388	443	373	1	0,4	0,8	0,3	
	467	598	439	3	1	2,08	0,8	
	527	654	472	7	1,5	2,5	1,05	
	554	677	448	21	1,7	2,7	0,9	
3T4	238	345	412	0	-0,7	0,06	0,6	
	365	538	565	1	0,2	1,6	1,8	
	385	691	767	3	0,4	2,8	3,5	
	440	786	851	7	0,8	3,6	4,2	
	867	771	1001	21	4,3	3,5	5,53	

OT0	a	b	c				
	251	464	324	0	-0,65	0,99	-0,09
	401	605	478	1	0,5	2,1	1,1
	439	774	657	3	0,8	3,5	2,56
	357	928	736	7	0,15	4,88	3,2
	600	1039	868	21	2,1	5,88	4,35
	6617	2042	512	0	87,6	15,99	1,37
	6700	2036	917	1	89,3	15,92	4,78
	6792	734	771	3	91,2	3,2	3,5
	6907	991	736	7	93,6	5,45	3,2
	6819	1126	2013	21	91,7	6,67	15,67

Table 8
cont.zero
control
experiment
results

Table 9 data for Calibration

Area	ppm		
216	0,3	310	0,3
870	1,6	500	1,6
1557	4,6	1060	4,6
1786	6,6	1540	16
2375	13,2	2640	33
3207	33	4020	82
4490	66	6740	163

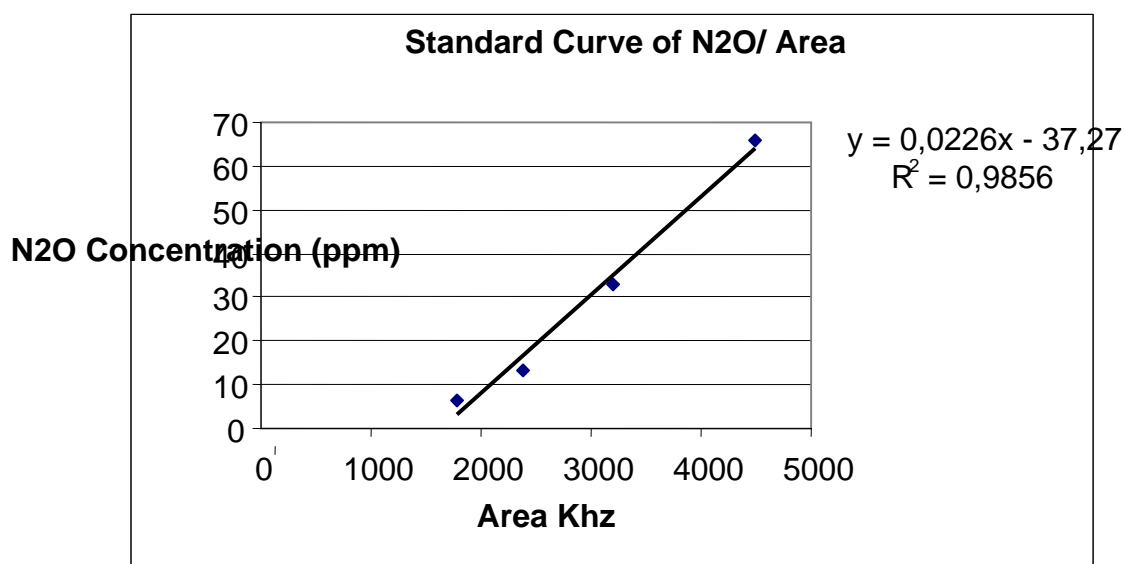


Figure 12a

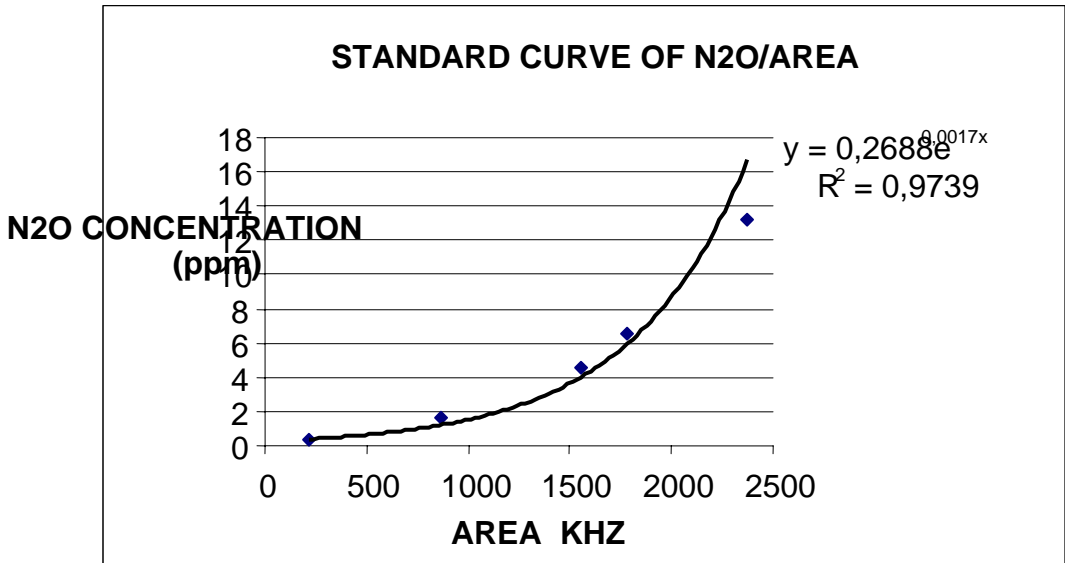


Figure 12b

OT0 Trt	A (Area)	y1	B (Area)	y2	X (Time)	Y1 N2O	Y2 N2O
0	2793	25,9	2063	9,35	0	25,9	9,35
1	1888	6,59	1864	6,33	1	6,59	6,33
3	2871	27,6	2622	21,98	3	27,6	21,98
7	3424	40,1	3409	39,77	7	40,1	39,77
21	1598	96	1609	98,88	21	96	98,88
23	1866	154	1734	117	23	154	117
1T3							
0	1301	2,4	1430	2,96	0	2,4	2,96
1	1644	4,4	1408	2,93	1	4,4	2,93
3	2062	8,9	1101	1,43	3	8,9	1,43
7	2688	23,5	1191	2,02	7	23,5	2,02
21	4650	67,8	3536	42,6	21	67,8	42,6
23	1381	64,32	3732	47,07	23	64,32	47,07
1T4							
0	1491	3,27	1352	2,65	0	3,27	2,65
1	1652	4,4	1448	3,14	1	4,4	3,14
3	2053	8,8	1948	7,28	3	8,8	7,28
7	2625	22,05	2599	21,46	7	22,05	21,46
21	4748	70	4599	66,7	21	70	66,7
23	1273	56	4804	71,3	23	56	71,3
2T3							
0	1737	5,1	1749	5,23	0	5,1	5,23
1	1964	7,5	1909	6,59	1	7,5	6,59
3	2696	23,6	2655	22,7	3	23,6	22,7
7	3387	39,27	3286	36,99	7	39,27	36,99
21	1816	140	4564	65,87	21	140	65,87
23	1792	134	1883	156,7	23	134	156,7
2T4							
0	1838	6,08	1877	6,52	0	6,08	6,52
1	1861	6,33	1962	7,51	1	6,33	7,51
3	2620	21,9	2697	23,68	3	21,9	23,68
7	2957	29,5	3355	38,55	7	29,5	38,55
21	1857	150	1806	138	21	150	138
23	1786	133	1856	150,5	23	133	150,5
3T3							
0	1794	5,67	1832	6,02	0	5,67	6,02
1	1976	7,7	2029	8,38	1	7,7	8,38
3	2624	19	2800	22,01	3	19	22,01
7	3352	29	3437	32,4	7	29	32,4
21	4320	60,36	1707	117	21	60,36	117
23	1886	158	1861	152	23	158	152
3T4							
0	1267	2,3	1762	5,34	0	2,3	5,34
1	1315	2,49	2018	8,29	1	9	8,29
3	939	1,31	2792	25,83	3	17,1	13,83
7	935	1,3	3462	40,97	7	18,3	21,97
21	2512	19,5	1729	117	21	19,5	117
23	2665	22,95	1931	171	23	22,95	171

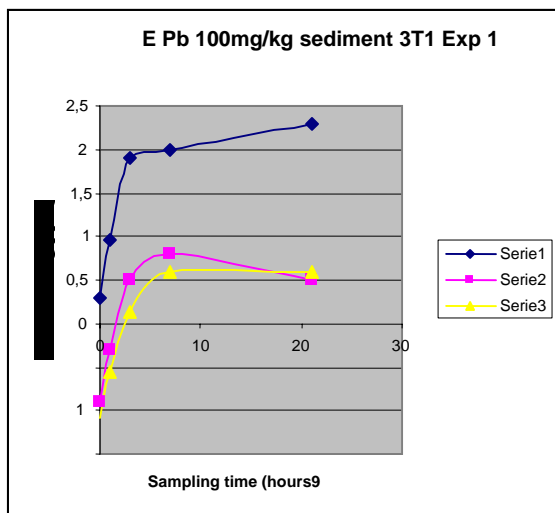
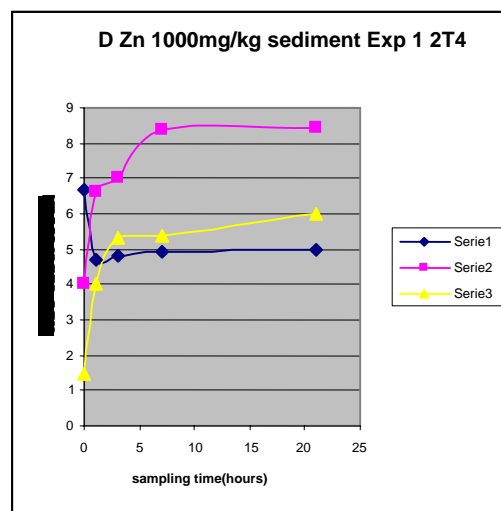
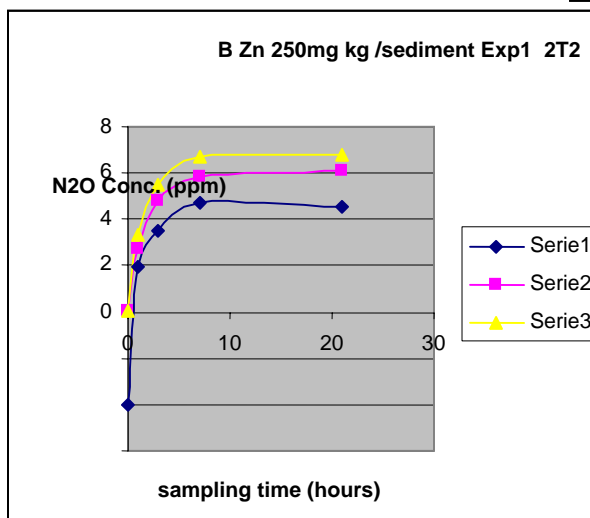
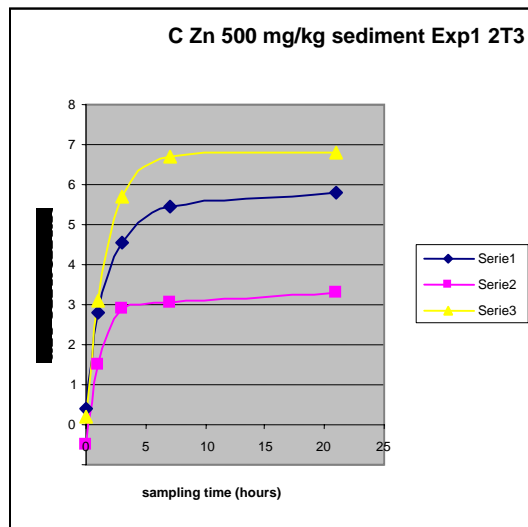
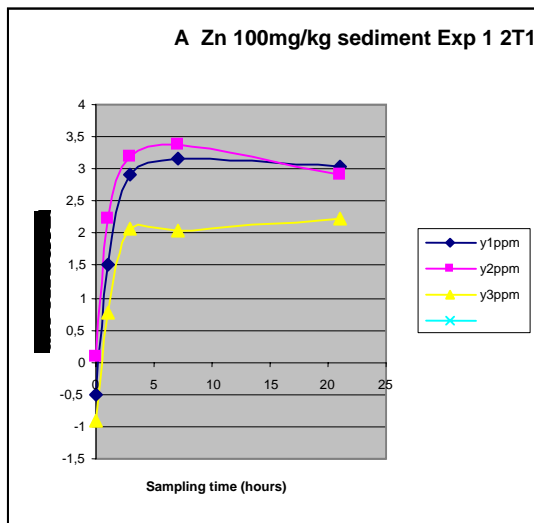
Table 10 Results of experiment 2, Cu, Zn and Pb treatment at 500mg/kg and 1000mg/kg of sediment

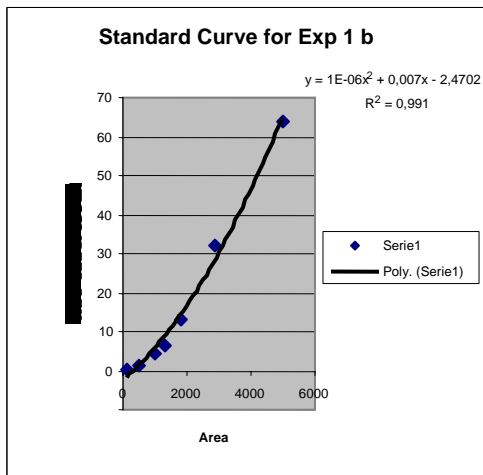
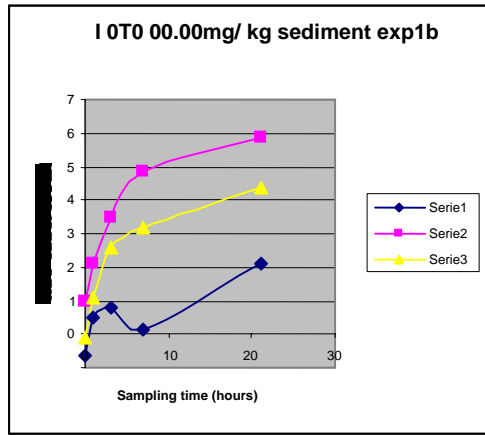
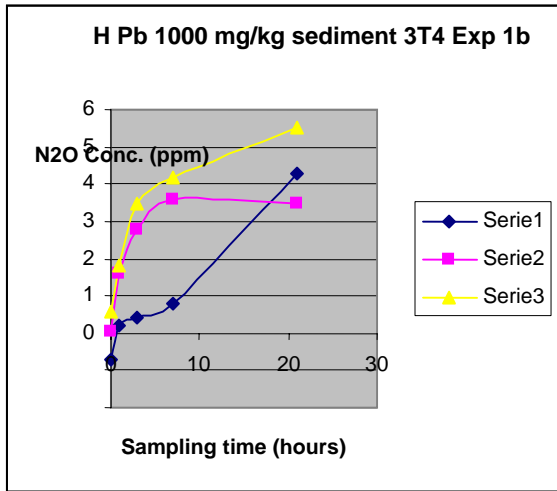
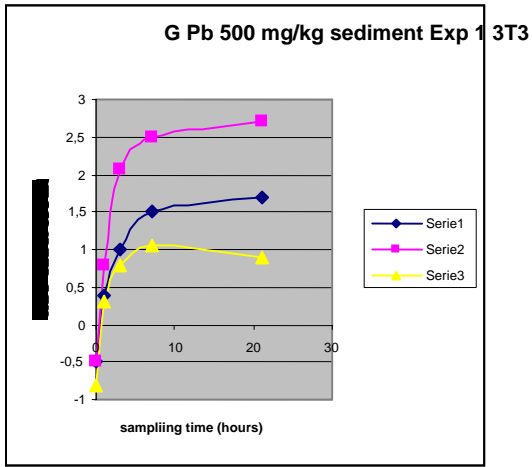
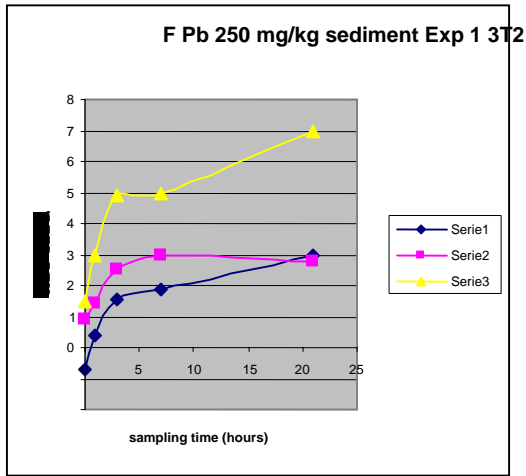
Table 11 Data set for the multiple regressions

N2O	Time Hrs	T*t1	T*t2	T*t3	Copper	Zinc	Lead
7	0	0	0	0	0	0	0
26	1	0	0	0	0	0	0
28	3	0	0	0	0	0	0
40	7	0	0	0	0	0	0
6	0	0	0	0	0	0	0
9	1	0	0	0	0	0	0
22	3	0	0	0	0	0	0
40	7	0	0	0	0	0	0
2	0	0	0	0	1	0	0
4	1	1	0	0	1	0	0
9	3	3	0	0	1	0	0
24	7	7	0	0	1	0	0
3	0	0	0	0	1	0	0
3	1	1	0	0	1	0	0
8	3	3	0	0	1	0	0
20	7	7	0	0	1	0	0
3	0	0	0	0	1	0	0
4	1	1	0	0	1	0	0
9	3	3	0	0	1	0	0
22	7	7	0	0	1	0	0
3	0	0	0	0	1	0	0
3	1	1	0	0	1	0	0
7	3	3	0	0	1	0	0
22	7	7	0	0	1	0	0
5	0	0	0	0	0	1	0
8	1	0	1	0	0	1	0
24	3	0	3	0	0	1	0
40	7	0	7	0	0	1	0
5	0	0	0	0	0	1	0
7	1	0	1	0	0	1	0
23	3	0	3	0	0	1	0
37	7	0	7	0	0	1	0
6	0	0	0	0	0	1	0
6	1	0	1	0	0	1	0
22	3	0	3	0	0	1	0
30	7	0	7	0	0	1	0
7	0	0	0	0	0	1	0
8	1	0	1	0	0	1	0
24	3	0	3	0	0	1	0
38	7	0	7	0	0	1	0
6	0	0	0	0	0	0	1
8	1	0	0	1	0	0	1
22	3	0	0	3	0	0	1
32	7	0	0	7	0	0	1
6	0	0	0	0	0	0	1
8	1	0	0	1	0	0	1
19	3	0	0	3	0	0	1

29	7	0	0	7	0	0	1
2	0	0	0	0	0	0	1
9	1	0	0	1	0	0	1
17	3	0	0	3	0	0	1
18	7	0	0	7	0	0	1
5	0	0	0	0	0	0	1
8	1	0	0	1	0	0	1
13	3	0	0	3	0	0	1
22	7	0	0	7	0	0	1
6	0	0	0	0	0	0	0
17	1	0	0	0	0	0	0
24	3	0	0	0	0	0	0
37	7	0	0	0	0	0	0
7	0	0	0	0	0	0	0
22	1	0	0	0	0	0	0
23	3	0	0	0	0	0	0
38	7	0	0	0	0	0	0

APPENDIX 2 Graphical results (linear regression) of Zn and Pb treatment experiment 1b.





j

Figures 7a-j: graphical representation of results in the Zn and Pb treatment in experiment 1b which showed less response

Appendix 3

TREATMENT PREPARATIONS

We prepared the metal salt solution based on the calculation of dried weight samples made equivalent to the treatment level of 1000 mg/kg sediment for each of the samples: Cu, Zn and Pb. Further deductions were half of the 1000 mg/kg i.e. $1/2 = 500$ mg, $1/4 = 250$ mg, and $1/10 = 100$ mg.

A 1ml equivalent to 1000 mg/kg, 0.5ml gives 500 mg, 0.25ml = 250 mg and 0.1 ml = 100 mg.

Dried weight of sediment 5ml estimated as 2.07g
1000 mg for 1kg of sediment
X mg then 2.07g

$X \text{ mg} = 1000 \times 2.07/1000 = 2.07 \text{mg/ml}$ standard conc. for 2.07mg as equivalent to 1000 mg/kg= 1ml

To prepare 25ml with this standard, $25 \times 2.07 \text{ mg} = 51.75 \text{ mg/ 25ml}$.

Cu Cl₂. 2H₂O (0.03M)

Molecular weight =170.48g/ mol

Cu = 63.52g/mol

51.75 mg is equal to $51.75 \times 1000 \times 170.48 / 63.52 \times 1000 = 139 \text{mg}$ (impure) Cu Cl₂. 2H₂O

139 mg in 25 ml i.e. 5560 mg/L Thus conc. In mole $5560/170.48 \times 1000 = 0.03 \text{M}$

ZnCl₂ (0.03M)

Molecular weight 136.29g/mol.

Zn

65.39g/mol

Cl₂ 35.45x2/mol

$136.29 \times 1000 \text{ mg impure} = 65.39 \times 1000 \text{ mg of pure Zn}$

X mg of impure Zn in 51.75 mg

X mg

$= 136.29 \times 1000 \times 51.75 / 65.39 \times 1000 = 107.85 \text{mg/25ml}$ standard treatment solution for Zn.

4314 mg/L which gives 0.03M

PbCl₂ (0.01M)

278.10g/mol

Pb 207.2g/mol

Cl 35.45x2 g/mol

$278.10 \times 1000 \text{ mg PbCl}_2 = 207.2 \times 1000 \text{ mg of Pb}$

X mg of impure PbCl₂ in 51.75 mg pure Pb

$X \text{ mg} = 278.10 \times 1000 \times 51.75 / 207.2 \times 1000 = 69.45 \text{ mg of PbCl}_2 / 25 \text{ml of the Pb}^{++} \text{ solution.}$

2778mg /1000ml equivalent to 0.01M

3b. The concentrations used.

Chemicals	MW (g/mol)	Final concentration mM
Buffer KH_2PO_4 (w1)	136.09	3 (0.4g/l)
Na_2HPO_4 (w2)	141.96	3 (0.4g/l)
Minerals w3		
NH_4Cl	53.49	5.6 (0.3g/l)
NaCl	58.44	5.1 (0.3g/l)
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	147.02	0.8 (0.1g/l)
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	203.30	0.5 (0.1g/l)
Trace mineral w5		
$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$	198.	10 (2000ug/l)
H_3BO_3	61.	0.8 (50ug/l)
ZnCl_2	136.	0.4 (50ug/l)
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	170.	0.2 (38ug/l)
$\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$	161.	0.3 (50ug/l)
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	1235.	0.3 (50ug/l)
AlCl_3	133	0.4 (50ug/l)
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	237.	0.2 (50ug/l)
$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	237	0.2 (50ug/l)
$\text{EDTA}(\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8 \cdot 2\text{H}_2\text{O})$	372	1.3 (500ug/l)
Nitrate: KNO_3	101.1	1.4mg/l

N/B W1, W2 W3 and W5 *Laboratory standard solutions* (TEMA VAT TEN) Linköpings University.