toxicity of 2-(nitroamino) ethanol and dimethylnitramine exposed to plants and microorganisms.

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Master Thesis 60 credits 2012

Toxicity of 2-(nitroamino) ethanol and dimethylnitramine exposed to plants and soil microorganisms

Abstract

Global warming is becoming one of the largest threats to the planet earth. The main cause of this problem is the emission of greenhouse gases from fossil. To minimize it the intergovernmental panel on climate change (IPCC) has urged to decrease global greenhouse gas emissions by 50 to 85 percent (Aarrestad & Gjershaug 2009). Carbon capture and storage (CCS) is proposed to be one of the best ways to reduce the impact of CO_2 emissions (Kristoffersen et al. 2008). But another concern of amine emission to the environment through Leakage and waste from the Carbon Capture and Storage has been raised proved (Aarrestad & Gjershaug 2009; Knudsen et al. 2009). The degradation products of amines, like amides and nitrosamine are known to be carcinogenic and mutagenic to animals. Nitramines are also suspected to be toxic but not well studied. Their toxicity effect to terrestrial organisms also is not studied. Therefore this study conducted to fill the gap of knowledge of their toxicity effect to terrestrial organisms through characterizing the mode of action, determining toxic potency, species sensitivity and vulnerability at their different life stage and determining if the effect is likely to occur under realistic exposure scenarios. To answer these objectives we conducted an experiment by exposing amines to two plant species (*Helianthus annuus* and *Lolium multiflorum*) and microorganisms with dimethylnitramine and 2-(nitroamino) ethanol through soil matrix.

These experiments have been conducted according to the OECD guidelines No.208 and 227 for the plants (OECD 2006a; OECD 2006b) and OECD guidelines No.216 and 217 for microorganisms (OECD 2000a; OECD 2000b). From the seedling emergence and growth test, concentrations of 0.1- 94µmol/kg 2NAE were non-toxic to the two plant species during their growth stage. Seedling emergence was completely inhibited by 2NAE at concentrations of ≥ 942µmol/kg (100mg/kg) in both species. The weight of *L multiflorum* (fig. 6A) was about 50% of the control at 118 µmol/kg of 2NAE, whereas 471µmol/kg 2NAE 95% inhibited growth of *L. multiflorum*. In a similar manner, growth of *Helianthus annuus* was reduced by 50% at 118 µmol/kg and complete growth inhibition was observed at 942µmol/kg (100mg/kg).

But with plant vegetative vigour test and the microbial carbon and nitrogen transformation tests, both chemicals had positive effect. Both plant species had similar response for both compounds in the plant vegetative and vigour test with LOEC of 943 and 1110µmol/kg of 2NAE and DMNA respectively. The microbial carbon and nitrogen transformation were also stimulated starting from 1110µmol /kg of both compounds. The rough calculation of risk quotient on the observed adverse effect of 2NAE was less than 1. Therefore these compounds are not environmentally risky.

Preface

This master thesis was done under the EXSIRA Project (https://erom.niva.no.exsira) with collaboration between the Norwegian University of Life Sciences (UMB), the Norwegian Institute of Water Research (NIVA) and Bioforsk. The laboratory work was conducted at the Institutt for plante- og miljøvitenskap (IPM), the isotope and Bioforsk laboratory.

Acknowledgment

I would like to begin by sincerely thanking my main Supervisor, Prof. Knut Erik Tollefsen for having me in the project and his constant support, guidance and mentorship over the course of this thesis. He helped me to define my thesis statement and always he was very helpful. Whenever I was short of ideas, my discussions with him and his insights always helped me get back on the right track. He has always encouraged me to work hard widening the ideas. I have truly learnt a lot from him over the past one and half year and for this, I am very grateful.

I would also like to thank my co-supervisors Pro. John W Einset, and senior researcher Erik J. Joner (PhD.) for their valuable suggestions and advice and patience. It was real benefit and privilege working with you all.

Equally I would like to forward my heartily thanks to PhD. Claire Coutris for her mentoring, discussion and cooperation of the practical lab work and for helping analysing my data. I would like also to thank all the IPM Isotope laboratory and Bioforsk laboratory technicians.

Finally I really thank my whole family especially my Brother Pastor Meron D. Woldehawariat for his financial help and spiritual encouragement to finish this master work.

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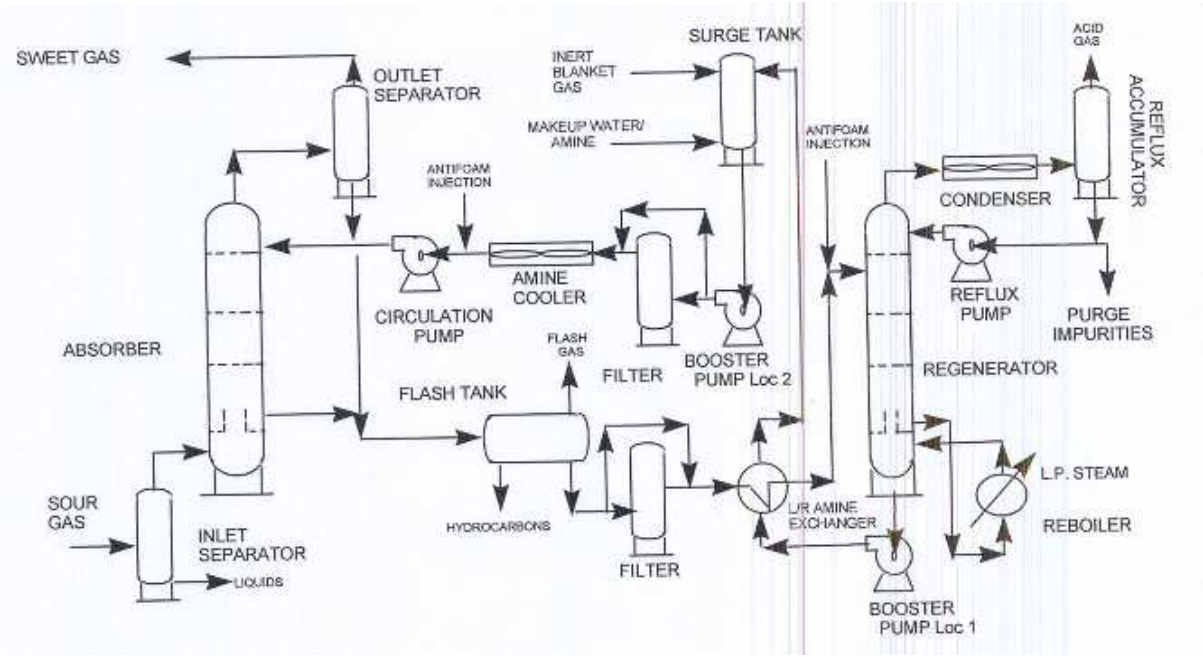
1. Introduction

1.2 Environmental challenges of Carbon Capture and storage

Our planet is experiencing large challenges in relation to increases in temperatures resulting from global warming scenarios. The main causes of the ongoing increases in global warming are the result of the emission of greenhouse gases such as carbon dioxide (CO_2) from burning of fossil fuels. If the present situation of greenhouse gas emission continues, temperatures will increase approximately by 5 degree Celsius in the 21 century (IPCC 2007 a). As a result, extinction of organisms, drought, and flooding and extreme weather condition may be considerably worse than seen today (IPCC 2007 a). To minimize these conditions, the inter-governmental panel on climate change (IPCC) has urged for a decrease in global greenhouse gas emissions by 50 to 85 percent during 2000 to 2050 (Stangeland 2007). As one possible solution to this problem, carbon capture and storage (CCS) is proposed to be one of the best ways to reduce CO_2 emission from combustion of fossil fuel (Kristoffersen et al. 2008). Carbon capture and storage technique captures transport and stores CO_2 by performing post-combustion CO_2 capture. In post-combustion CO_2 capturing (fig. 1), CO2 is separated by absorption using chemical solvents such as amines (Kristoffersen et al. 2008). The CO_2 separated from the amines by heating and the amines are then recycled for use to the process again.

Throughout the whole process of capturing and storing of CO_2, there are emissions of amines and their degradation products to the atmosphere. Some studies (Knudsen et al. 2009; Mascher et al. 2005a) have suggested that there is a potential for escape of amines from CCS facilities causing effects on organisms in the environment around the CCS plants.



(F. & Bosen)

Figure 1. Principle of carbon capture using amines: the flowing gas from the power plant and the amines with water from the top of absorber meets in the absorber column. The amines capture the CO_2 by making chemical bond inside the absorber and form carbonate. Then the carbonate heated to separate the co_2 from amines. Then the separated CO_2 transport to storage site but the amines recycled into the absorption process again

Little data have been published on the toxicity effects of amines in the environment despite their wide-spread use and potential toxicological effects on terrestrial organisms are virtually uncharacterized. The present study thus aims to increase the knowledge of amine toxicity to terrestrial plants and microorganisms exposed to the two amines dimethylnitramine (DMNA) and 2-(nitroamino) ethanol (2NAE).

1.2 Amines

Amines often used in CCS processes are chemicals that are derivatives of ammonia in which one or more of the hydrogen atoms has been replaced by an alkyl or aryl group (Blauwhoff et al. 1984). Amines are classified as primary, secondary, or tertiary depending on whether one, two, or three of the hydrogen atoms of ammonia have been replaced by functional groups.

Some of the amines most commonly used in CO2 capture are monoethanolamine (MEA), methyldiethanolamine (MDEA), 2-Amino-2-methylpropanol (AMP), Piperazine (PIPA), diglycolamine (DGA), diethanolamine (DEA), and di-isopropanolamine (DIPA). The chemical structures of these amines are shown in Figure 2

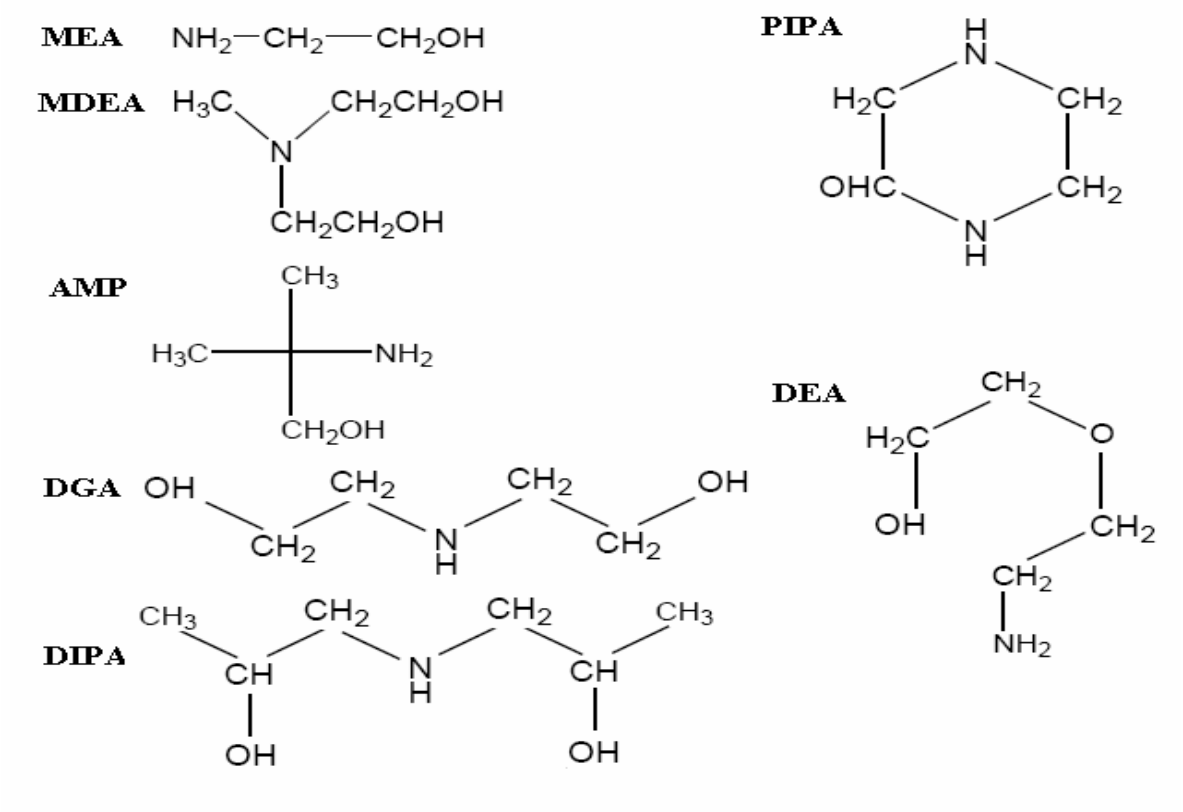


Figure 2. Chemical structures of the amines most commonly used in CO2 capture: Monoethanolamine (MEA), methyldiethanolamine (MDEA), 2-amino-2-methylpropanol (AMP), Piperazine (PIPA), diglycolamine (DGA), diethanolamine (DEA), and di-ispropanolamine (PIPA).

In newer technologies, the solvents used for CO2 capture are commonly a mixture of several different amines. This mixture of amines includes blends of MEA-piperazine, MDEA-piperazine blends, and blends of N-methyldiethanolamine (MDEA) and triethylene tetramine

(TETA). For several new technologies, the amine mixtures are not publicly known because the solvent suppliers regard this as proprietary information. The amines used for CO2 capture are recycled, but a minor portion of the amines are either degraded or emitted to air. These emitted amines are often unstable in nature, and discharged amines may degrade to hazardous substances that are toxic and may thus represent a risk to different organisms. Such degradation products include aldehydes, amides, nitrosamines; nitramines (*cf. Figure* 3 for chemical structure). Amines and their degradation products can be hazardous to human health, animals, plants and to the environment (Aarrestad & Gjershaug 2009).

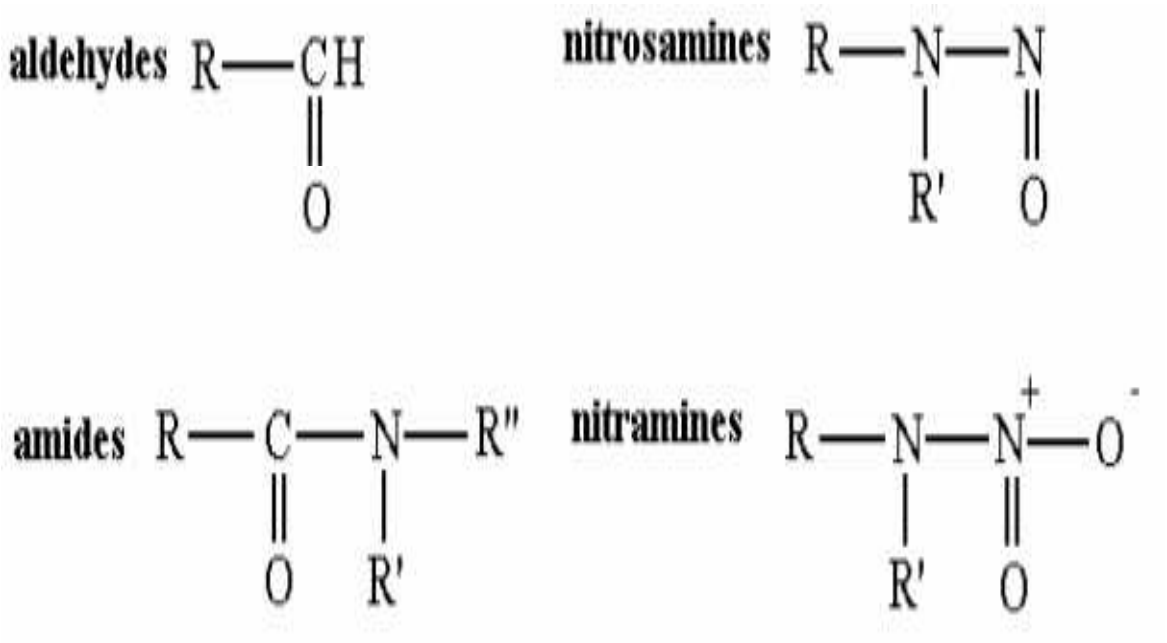


Figure 3. General chemical structures of the atmospheric degradation products of amines

The greatest concern in CCS is the atmospheric emission and distribution of amines and amines degradation derivatives to environment. During the overall process of CCS, amines and their derivatives might escape to the environment through emission, leakage and as waste products (Aarrestad & Gjershaug 2009; Kristoffersen et al. 2008). The escaping of amines to the environment small fraction from the absorber as purified exhausting gas (Kristoffersen et al. 2008), and from scrubber water droplets as gas emissions. Emission of these amines from CCS plants depends on the concentrations of the components from which they are derived from.

From the four degradation products of amines (aldehydes, amides, nitrosamines and nitramines) formed in the carbon capture process, nitramines and nitrosamines are of main concern because they are suspected to be carcinogenic. Theoretically, nitrosamines can be formed from secondary and tertiary amines but most significantly from tertiary amines (Låg et al. 2011). The formation of nitrosamine in the aqueous solution depends on the pH and concentration of nitrogen oxide

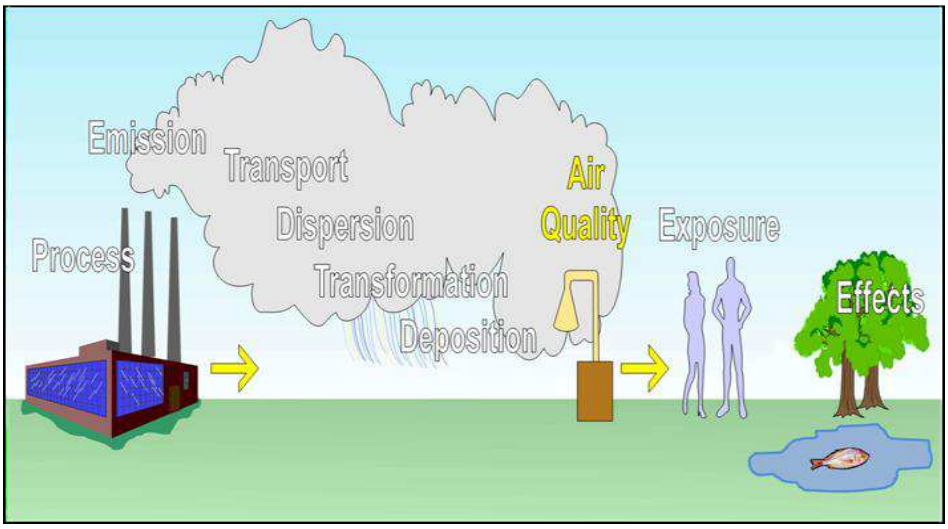


Figure 4. Pathway of amines through the atmosphere: from emission to exposure/load and effects/risks. Amines after emitted from the power CCS plant, it transport and transform in the air and disperse to the ecosystem exposing animals and plants in terrestrial as well as in water bodies (Knudsen et al., 2009).

For instance, the estimated amine emission expected from CO_2 capture plant at Kårstø (Norway) is 40 to 160 tons per year (Kristoffersen et al. 2008). The exact amount will depend on the size of the power plant, but represent a fairly large amount if expected to become deposited to the terrestrial environment.

The second way of amine dispersion from the CCS to the environment can be through waste. In the carbon capture storage process the major waste will be the water coming from the reclaimer, e.g. the separation of the usable amine and its degradation products (Knudsen et al. 2009; Kristoffersen et al. 2008). The function of the reclaimer in the CCS is to avoid accumulation of degraded amines from the CCS processes. It works by separating usable amines (which can be recycled to be used in the absorber again) and the degradation products. The waste contains amines, amine degradation products, water, corrosion products and other chemicals. The constituent of the waste depends on the type of gas used in the capture process and on the pH of the wash water.

The third way of contamination can be through possible leakages from the absorber, stripper, and reboiler. The reason for amine leakage from the absorber, stripper, and reboiler is is often due to corrosion (Kristoffersen et al. 2008). Waste water is the main cause of amine emissions and liquid droplets from the flue gas contribute a small amount. Most of emission is expected to occur with neutral water washes as the amines readily make the water basic whereas acids neutralize the amines in the wash water.

Worst-case scenario estimation of deposition of amines from emissions of solvent and flue gas as guideline for health based on TCM (technology center Mongstad) are 0.3ng/m3 in air and 4ng/m3 in drinking water (Låg et al. 2011).

These estimates are not obtained by direct measurements of nitramines and nitrosamines but from air amine degradation through hydroxyl (OH•) radical reactions during day and reductions with other radicals (NO_2 •) during the night (Nielsen et al. 2010). Amines such are dimethylnitramine (DMNA) and 2-nitroaminoethanol (NAE), are among the degradation products of amines.

1.2.1 Physicochemical properties

Understanding the physicochemical properties of chemicals is very important for predicting mobility, transformation, accumulation, magnification and toxic potential of the pollutants in the environment (Miyamoto & Klein 1998). Amines can be divided into aromatic and aliphatic amines, depending on their functional groups. The main used amines in CCS are the aliphatic amines and they are considered to be strong organic bases (Nelson 1985).

The basic or hydrolysis characteristic of the lower aliphatic amines is mainly due to their nucleophile character, resulting from the presence of an unshared pair of electrons on the nitrogen atom (Kawa et al. 1992; Solomons et al. 2000). The properties of amines are largely controlled by the electronic characteristics of the electron pair on the central nitrogen atom (or atoms for bi- and poly-functional amines), which is able to act as a Lewis Base and use the lone pair of electrons for electron donation. The ability of the nitrogen atom to donate its lone pair of electrons in chemical reactions is modified by the presence of the functional

groups bonded to the nitrogen atom that can increase or decrease this ability. Due to their basic character, they are used as neutralizer or pH stabilizers.

These compounds are colourless gases or highly flammable liquids with strong odours. The lower molecular weight molecules, with low carbon contents are water soluble and sold as pure salts. Amines are hygroscopic, they have the ability to hold or grasp water molecules from their surrounding environment (Bråten; H.B. et al. 2009). They react with water and acid and form alkyl ammonium compounds. The base strength in water for primary; secondary, tertiary amines are similar to ammonium. Alkyl amines are corrosive to copper, copper-containing alloys (brass), aluminium, zinc, zinc alloys, and galvanized surfaces. Aqueous solutions of alkyl amines slowly etch glass as a consequence of the basic properties of the amines in water (Smith 1965).

Secondary amines react with nitrous acid or nitrogen oxide present in air to form nitrosamine ($R_2NH + HNO_2 = RN_2\text{-}N{=}O$) (Smith 1965). Amines also react with various substrates such as epoxides, aldehydes and ketones, alkyl halides, carboxylic acids/halides/esters/anhydrides, and carbon disulfide to produce useful products in agriculture (herbicides) and industrial applications, such as rubber chemicals, catalyst, detergents, textiles etc.

Table 1, Physicochemical of 2-(nitroamino) ethanol, dimethylnitramine and other CCS amines (Wittgens et al. 2010)

Name amines	Abbreviation	CAS.No.	Melting Point °C	Boiling Point °C	Aggregate State at room Temperature.	Solubility in water g/l pH7	Octanol water Partition coefficient LogP
2-ethanolnitramine	2NAE	7438-82-6	37-39	266	L	169	-0,806
Dimethylnitramine	DMNA	4164-28-7	58	187	S	176	-0,447

Monoethylamine	MEA	141-43-5	10	171	L	1000	-1,48
Diethylamine	DEA	111-42-2	28	268	S	1000	-1,761
Peperazin	PPZ	110-42-2	106	146	S	1000	-1,5
2-amino-2-methyl-1-propanol	AMP	124-68-5	30	165	S	1000	-0,716
N-methyl-di-ethanol-amine	MDEA	105-59-9	21	247	L	1000	-0,619

Chemicals such us 2NAE and DMNA are commonly used in explosive materials because they are very reactive to strong oxidizing agent. Both are highly soluble in water: 169g/l and 176g/l at room temperature respectively. They are stable under normal conditions and exist as liquid at room temperature. Both are white in colour when they are at their solid state. The most important physicochemical properties in characterizing their solubility, melting and boiling temperature and their measure of lipophilicity (octanol/water coefficient) are compiled in Table 1. Amines are miscible in water and since amines have low Koc values (Sorensen et al. 1997), they are not easily absorbed by organic carbon in soil.

1.2.2 Fate

1.2.2.1 Atmospheric degradation of amines

In the CCS process chemical degradation of amines occurs primarily through thermal and photochemical reactions (Knudsen et al. 2009). The thermal degradation of amines occurs at the striper, reboiler and reclaimer while the amines are heating to separate or unbinding the carbamate. The photochemical degradation also occurs after emission of the amines and takes

place outside the power plant in the air. Degradation of these solvents can also take place by sulfur oxides (SO_x) and nitrogen oxides (NO_x) reaction.

Other impurities in the exhaust gas such as fly ash also contribute to degradation of solvents. Low concentrations of a large number of degradation products could be formed through photochemical reactions in the atmosphere after emission from the CCS process (Bråten; H.B. et al. 2009). The main chemical properties affecting emission rates of the degradation products are volatility, base strength/acid strength and their affinity to other chemicals species present in the liquid. As most degradation products are ionic they should be expected to have low vapour pressure, and therefore not likely to be volatile.

According to simulation experiments, the main degradation of the solvents after emissions is through photooxidation reaction (Låg et al. 2011). Nitramines and nitrosamine are degradation products of the MEA (solvent) as a result of photooxidation reactions. Also, after deposition to soil, the amines might be subject to biological degradation in biotic environments. In all degradation reactions, deamination of the amine is the most important and ultimate reaction leading to production of aldehydes, alcohols and organic acids as putative products.

For example, dimethylnitramine is identified as volatile degradation products in the post CO_2 combustion processes and it is identified to form nitrosodimethylamine by reacting with gaseous nitrous acid in the presence of light (Hanst et al. 1977). 2-nitroaminoethanol was confirmed as a minor degradation product of MEA (Nielsen et al. 2010). But in the atmosphere nitramines are relatively more stable than nitrosamines, with half-life of 2 days for nitramine and half day for nitrosamine (Karl et al. 2012)

1.2.2.2 Biodegradation of amines in soil

Degradation of CCS amines is also expected to occur in the soil or in the aquatic environment. Although most of the degradation data on amines and their degradation products originate from atmospheric degradation, only brief information is available for the degradation of amines in other environmental matrices such as soil.

Microorganisms degrade amines to ammonia acetaldehyde, acetate, nitrite, and nitrate and nitrogen gas under aerobic and anaerobic condition. Nitrification and carbon transformation are main microbial activity. Degradation pathway of amines in soils by microorganisms is Nitrification, methanogenesis and sorption (Gallagher et al. 1997).

Most of CCS used amines and their atmospheric degradation products are biodegradable (Låg et al. 2011). In a recent study the metabolites *N*-(2-anilinoethyl) acetamide and *N*-acetyl-*N*_-henylpiperazine were detected during biodegradation of piperazine by a strain of *Mycobacterium* sp. (Adjei et al. 2007). Ammonium which is derivative of amines can be sorbed onto clay minerals. Sorption of chemicals onto soils with higher organic matter decrease rate of biodegradation by retaining the chemicals from being bioavailable

Most microorganisms need temperature, moisture for growth and temperature is the main factor because if the temperature decreased below 6˚C, transformation of organic matter decreases significantly. Clay and organic matter in soil affects the bioavailability by inhibiting chemical distribution (Chung & Alexander 1999).

1.2.2.3 Bioavailability

Mobility of substances in soil depends on the adsorption and desorption processes, the structure of the soil, the organic matter content of the soil as well as on partition coefficients. Temperature and vapour pressure affects the rates of diffusion of substances in soil. Moisture increases volatility by reducing the active surface adsorption while presence of organic matter and clay decreases volatility by increasing the concentrations of reactive surfaces. High solubility, volatility and the presence of moisture aid the bioavailability of substances by enhancing mobilities.

The concept of bioavailability is used in toxicity assessment as an adjustment or correction factor, describing the ability of the toxicant to be absorbed by an organism. This concept involves several assumptions about different bioavailability processes. Bioavailability determines the amount of given chemical in soil or sediment or inside an organism that can be expected to cause biological responses. The transport of bioavailable substances in soil as well as inside the organ or system of the test organism can be through diffusion.

Amines are miscible in water and have low Koc values (log-0.223 to -0.308)(Sorensen et al. 1997) and are therefore not easily absorbed by organic carbon in soil. For uncharged organic compounds, this means they are mobile in the subsurface. Amines are largely protonated and exist as cations within a typical environmental pH range, and tend to sorb with charged surface of clay minerals. As a result the distribution coefficient (Kd) will be controlled by the cation exchange capacity of the soil. Therefore, amines are relatively immobile in clay soils but they are more mobile in sandy soils with low cation electro-conductivities. Amines acts as weak bases in aqueous solutions thus the addition of amines to soil water systems increases the pH (Sorensen et al. 1997). Overall, amines can be leached easily from sandy soils but les from clay soils.

The primary amines with less than 5 carbons are highly water-soluble and volatile compound and their concentrations in the ambient air may be high (Lundh et al. 1991). Most aliphatic amines with less than 5 carbons have less soil and sediment adsorptionsm making them easily bioavailable and freely degradable. The aliphatic amines with more than 5 carbons are not easily transformable or soluble in water.

1.2.2.4 Bioaccumulation

Bioaccumulation of substances in cells, organs and organisms depends on the lipophilic properties of the substances. In soils it depends on the amount of organic matter and type of the soil texture and structure. Bioaccumulation potentials of chemical substances can be determined by their octanol-water partition coefficients (K_{OW}), usually expressed as logarithmic value which is the ratio of their solubilities in octanol (e.g. a surrogate for lipid) compared to water. Water soluble compounds have low K_{wo} compaired to lipid soluble compounds. If the log K_{wo} value of a substance is >5 according to the Stockholm convention the substance will be categorized as having a high level of bioaccumulation(Rodan et al. 1999). Dimetylnitramine and 2-(nitroamino) ethanol are water soluble and with low tendency to bioaccumulation (Table 1)

1.2.3 Toxicity

Terrestrial environments as a recipient are one of the main receivers of pollutants from the atmosphere, water bodies, spreading of sewage sludge containing pollutants and point

sources of pollution, via rain, rivers and air transportation. All soil dependent organisms including man may be exposed to toxic materials entering the ecosystem through the soil (Calabrese 1993). Therefore, soil matrix for assessing environmental pollutants in terrestrial ecosystem is a common strategy(Calabrese 1993).

Amines have vast structures with broad chemical characteristics and their toxicity effect also broadly differs from severe toxic to very less. For instance aromatic and nitrosamines are known to have adverse effect. Aromatic amines are known to be source of bladder cancer in humans (Reh et al. 2000; Zenser et al. 1998).

Some nitrosamines are carcinogenic and mutagenic such as N-Nitrosodimethylamine and N-nitrosodiethylamine (Andrzejewski et al. 2005; Benigni et al. 2008). Some nitramines are speculated to be carcinogenic. For example, explosive nitramine compounds such as RDX (1,3,5-Trinitroperhydro-1,3,5-triazine) and HMX (Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) are registered as environmental contaminant, especially RDX classified as human carcinogenic (Category C) (Benigni et al. 2008; U.S.DepartmentofHealthandHumanServices 2010).

In general amines and all their degradation products are known to be source of nitrogen which can be also sources of eutrophication and water acidification (Aarrestad & Gjershaug 2009). Nitrosamines due to their potency for carcinogenic can be regulated as toxic substances, but there is in general a lack of thorough knowledge about the toxicity of amines to terrestrial organisms, due to lack of thorough studies.

The other negative impact of emissions and deposition of chemicals form different anthropogenic activity is eutrophication. Eutrophication is undesirable vegetation growth as a result of nitrogen saturation loaded from the atmosphere through precipitation and wind.

Based in past studies amines are part of nitrogen cycle. Amines, through degradation processes dissociate into nitrogenous compound, such as acetyl amine, ammonium, nitrate, nitrite and nitrogen gas(Aarrestad & Gjershaug 2009).

Eutrophication in terrestrial lands triggers growth of biomass and helps nitrophilic plants to over-compete the nitrophobic plants. It creates unbalanced and decreased biodiversity and facilitates disease in nitrogen sensitive plants. As cumulative effect it also affects the biodiversity by increasing herbivores by increasing grasses and shrubs, in alpine and arctic region vegetation can be affected if nitrogen deposition exceeds 15kgN/ha per year(Aarrestad & Gjershaug 2009). This condition favours grasses and shrubs to over-compete lichen, mosses, and ever green shrubs. For instance, in Norway, the critical load of nitrogen to change heath lands into more grass-dominated vegetation are in the range of 10 to 20kgNkg^{-1}yr^{-1}. The background deposition level in Mongstad area is 8 to 13kgNkg^{-1}yr^{-1} which is partially exceed the lower critical load (Aarrestad & Stabbetorp 2010).

1.2.3.1 Terrestrial ecotoxicological testing

Soil is one of the fundamental parts of the natural environment. It is largely non-renewable. The major economies and sources of economies are dependent on soil. Soil has also ecological and social functions. Ecological functions are biomass production, protection of humans and environments, and reservoir of gene. It provides all necessary materials for biomass production. It protects humans and the environment through buffering toxicant between the atmosphere and ground water. Also it protects animals and vegetation by absorption and adsorptions toxic materials, because it is shelter of millions and millions

species of living organisms. But without balanced use and justified management its ecological function can end in threatening by anthropogenic activities.

Moreover the urban/industrial activities, agricultural production and all life sustainability of terrestrial ecosystems depend on the quality of soil. Basic ecosystem functions such as food web, niche, habitat, connectivity and biodiversity are highly interdependent. Because from individual to community, from species to population and from primary producers to higher predators as part of ecological balance needs healthy and fertile soils and waters. Therefore to maintain these functions from abnormal and external hazard, terrestrial ecotoxicological testing methods are very critical. Terrestrial ecotoxicological testing is very important in risk characterizing, as one part of ecological risk assessment, like problem formulation and analysis and should use standardize toxicity testing methods.

Standardized terrestrial toxicity tests include soil microbes, plants, invertebrates, birds, and mammals. But the scope of the present study uses only microorganisms and plants toxicity evaluation.

Plants and microorganisms work in interconnection to sustain the terrestrial environment. Plants are the primary producers and microorganisms are the architect in maintaining the soil texture and nutrients recycling. Plants produce organic matter from sunlight as sources of building block and sources of energy, and support heterotrophic organisms. Microorganisms as heterotrophic in turn provide nutrients by breaking down raw organic matter and nitrogen for plants through nitrification processes in the soil root zone. They are terrestrial

representative and sensitive to exposure (Whitford 1989). Plants as well as microorganisms are very important as biomarkers for terrestrial ecosystem.

A range of species Perennial ryegrass (*L. multiflorum)* and sunflower (*H. annuus) (OECD 2006a; OECD 2006b)* as well as the soil microorganisms as intact communities(OECD 2000a; OECD 2000b) used to assess chemicals adverse effects spelled into soil.

Ryegrass and sunflower are terrestrials angiosperms, presenting dicotyledons and monocotyledons (Markwiese et al. 2001). Ryegrass and sunflower are one of the recommended plants for toxicity test in the OECD guidelines (OECD 2006a; OECD 2006b). Ryegrasses can be used as important biomarker due to its sensitivity towards toxicants and over fertilization or eutrophication as nutrient loving plant. Both species are also main portion of the animal feed which makes them crucial in terrestrial toxicity assessment. Additionally, Sunflower is one of the heavy feeder plants; it has potential in bioremediation and also plays big role in food web (Krizkova et al., 2008).

The seedling emergence and seedling growth test and the vegetative vigour tests(OECD 2006a; OECD 2006b) also designed to assess if chemicals has any adverse effect on plants. Seedling emergence and growth test can be conducted by spiked and mixed the chemicals in the soil before planting of the seeds. Seeds placed in the contaminated soil with the test substance evaluated for rate of germination and growth change of the seedlings comparing with the untreated control samples. Measurement takes place after 14 to 28 days of exposure after 50% emergence of seedling in control sample. Endpoints measured are visual

assessment of seedling emergence, biomass (dry weight or fresh weight) and visual detrimental effects (Chlorosis, mortality, and development abnormality).

The vegetative vigour test is also aimed to determine if a chemical exerts adverse effect on the plant during its development stage exposed on leaves and above ground portion of the plants applied as foliar deposition after the emergence of 2 to 4 leaves. The endpoints are change in biomass and visual detrimental effects such as chlorosis, mortality, and development abnormalities.

Microorganisms (OECD 2000a; OECD 2000b) are also among the recommended organisms in the OECD guide lines for assessing risks associated with identified chemicals. Investigating the changes in microbial activity contributes importantly to toxicity assessment. Any long-term interruptions of these biochemical processes will also affect the fertility of soil by interfere with nutrient cycling and soil fertility. If the soil is free from any harmful interference of contaminants, microorganisms will not cease metabolic activities: carbon and nitrogen transformation from organic matter.

Therefore the OECD guidelines no.216 and 217 (OECD 2000a; OECD 2000b) are meant to detect the adverse effect of the contaminants on microorganisms' by measuring there processes of carbon and nitrogen transformation in the long-term as inhibition and stimulation of the microbial activities. If the rate of change of these carbon as well as nitrogen transformation activity of microorganisms decreased in the treated samples from the control it implies an adverse effect of the compounds. The data from these tests give the dose

response curve and the effective concentrations. Therefore the end points in these tests are the change of nitrogen and carbon transformations as indicators of the activity and growth of soil microorganisms.

According to the OECD (Organisation for Economic Co-operation and Development) test Guidelines 216 and 217(OECD 2000a; OECD 2000b) and ISO (International Organisation for Standardization) test method 14238 (ISO, 1997) the soil moisture content is sensitive and it should be maintained between 40% and 60%.

1.2.4 Environmental risk assessment

The purpose of environmental risk assessment (ERA) is to protect the environment form chemicals causing adverse effects, and determined its safety and compatibility to the environment. If the predicted no effect concentration (PNEC) i.e. the concentration that causes no adverse effect to the environment organisms is higher than the predicted environmental concentration, which is predicted to be present in the environment then the chemical has risk. Therefore the PEC/PNEC ratio indicates the state of risk, called risk quotient. And if PEC is less than PNEC the substance is safe to the environment, because the ratio of PEC to PNEC or PEC/PNEC will be less than 1.

The ERA is a step wise processes, which goes from screening experiment and conservative assumption to realistic experiment and realistic assumptions(ECB 2003).

The first step is calculating the predicted environmental concentration: short term and long term exposures. Short term exposure means high concentration in short period of time (acute) and long term exposure is low concentration but for long period of duration (chronic). During exposure assessment understanding of biodegradability (persistency of the chemicals in atmosphere, air soil and water is very crucial. The next step is choosing sensitive species to indicate its harm. In the present study we select two plant species and soil microorganism according the OECD guidelines described in section 3. The third step is conducting dose response test using the selected species and finally characterizing the risk by calculating the risk quotient. To get reliable Risk Quotient the result (NOECs) of the end points must be generated from different species, which are representative of the ecosystem with different trophic levels (ECB 2003).

2. Objectives

The objective of this study was to assess the toxicity of the amines 2NAE and DMNA in a test battery of terrestrial plants and microorganisms through:

- Characterizing toxicity mode of action
- Determine toxic potency
- Comparing to species sensitivity and vulnerability of different life stages
- Determine if effects are likely to occur under realistic exposure scenarios

3. Materials and methods

The methods used in this study were based on the OECD guidelines developed for testing chemicals using plants and microorganisms:

a) Adverse effects on plants by conducting the OECD Guideline 208 “Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test” and the OECD Guideline 227 “Terrestrial Plant Test: Vegetative Vigour test”(OECD 2006a; OECD 2006b).

b) community by the OECD Guideline 216 “Soil microorganisms: Nitrogen Transformation Test” and OECD Guideline 217 “Soil microorganisms: Carbon Transformation Test” ((OECD 2000a; OECD 2000b).

3.1 Chemicals and solutions

The test chemicals and reference chemicals were diluted in deionised water prior to mixing into the soil. Each replicate soil sample was added a fixed volume of dissolved chemicals at an appropriate concentration.

- Distilled water ($_dH_2O$)
- Boric acid (H_3BO_3, CAS No. 10043-35-3, 98% purity, Sigma-Aldrich, Saint-Quentin Fallavie, France)
- D-(+) Glucose anhydrous ($C_6H_{12}O_6$, CAS no. 492-62-6, 99.5% purity, Sigma-Aldrich)
- L- Glutamatic acid ($C_5H_8NNaO_4 \cdot H_2O$, CAS no. 6101-04-3, Sigma-Aldrich).
- Cadmium sulfate ($3C_dS_{O4} \cdot 8H_2O$, 99% purity, Merck, Darmstadt, Germany)

- Dimethylnitramine ($C_2H_6N_2O_2$, CAS. No. 4164-28-7, 98% purity, Chiron, Trondheim, Norway)
- 2-(nitroamine) ethanol ($C_2H6N_2O_3$, CAS No. 74386-82-6, 98% purity, Chiron)

All chemicals except amines were stored at room temperature but the amines were stored in dark covered by black papers at 4◦C.

3.2 Organisms and culturing

The two plant species prescribed by the OECD test guidelines (*H. annus* and *L. multiflorum)* were planted as seeds. Clover (*Trifolium subterraneum*) was cultured in a greenhouse at University of Life Sciences (UMB) and its green biomass, freshly dried and used as substrate for the Nitrogen transformation test. *L. multiflorum* was supplied as seeds by Herbiseed (Twyford, England) and *Helianthus annus* from private grower (Ås, Norway). Microorganisms were collected as topsoil according to the OECD guidelines(OECD 2000a; OECD 2000b) from an organic field trial with wheat at Vollebekk (Ås, Norway) and assayed as an indigenous community.

3.3 Exposure matrices

3.3.1 Soil composition

The test matrix for both plants and microorganisms was soil collected and sampled at Vollebekk experimental Farm (Ås, Norway), diluted with sand from Askania (Drøbak, Norway) to achieve the prescribed organic matter content between 1% and 1.5 % according to the OECD guidelines (OECD 2000a; OECD 2000b; OECD 2006a; OECD 2006b). The farm from which the soil samples were collected was free from any agrochemicals (pesticide and fertilizers) and no organic manure was added to this soil for the last six months before the collection of the soil samples.

The objective of mixing the sand with the farm soil was to obtain a soil with 70 to 75 % sand, 20 percent clay and 0.5 to 1.5 % organic matter(OECD 2000a; OECD 2000b; OECD 2006a; OECD 2006b). The organic matter content of the soil sample before mixing with sand was between 3 to 5 % and was measured by dry ashing in an oven at 550 °C(Heiri et al. 2001). The organic matter content of the final soil medium was also calculated after measuring the weight of the soil before and after dry ashing in an oven at 550 °C (Heiri et al. 2001). The maximum water holding capacity of the soil-sand mixture (hereafter referred to as "soil") was measured by filling a 10 cm plastic cylinder with soil and saturating it with water. After draining into a sand bath for 8hours, a sample was taken and dried at 105 degree C and the percentage of maximum water holding capacity was calculated (ISO11465 1993). The pH and electric conductivity of the soil was measured in a soil: water suspension (1:5), w/w using a pH meter (ISO10390 1994). Table 2 summarises characteristics of the soil used for plant and bacterial toxicity tests

Table 2 Parameters of the soil matrix

Soil Samples	W.W. (g)	D.W. (g) @ 105◦c	D.W.(g) @550◦c	M.W.H.C (%)	pH	EC (mV)	TOM. (%)	TOC (%)
Sample 1	53.87	42.80	42.18	25.8	6.63	11.40	1.46	0.85
Sample 2	42.20	33.22	32.69	27	6.61	11.01	1.62	0.94
Sample 3	52.92	41.57	40.82	27	6.63	11.61	1.83	1
Average	49.66	39.20	38.56	26.60	6.62	11.34	1.63	0.93
SD	6.48	5.21	5.13	0.01	0.0	0.30	0.30	0.186

3.4 Experimental approaches

3.4.1 Reference Chemicals

As positive controls, we used boric acid for plant and cadmium sulphate for microorganism tests. Both, boric acid and Cadmium sulphate prepared by mixing with deionized water and diluted as logarithmic concentration. The purpose was to measure the acceptability of the test and to provide a basis for comparisons for interpreting the results and to make sure the soil medium is good to expose the testing organisms with testing chemicals.

Initially we run (Boric acid: 2444µmol/kg and 6110µmol/kg) for both plant species and (cadmium sulphate: 0.65µmol/kg and 0.13µmol/kg) for nitrogen and carbon transformation test. Then we used 2444µmol/kg boric acid in all plant test and 0.65µmol/kg cadmium sulphate for nitrogen and carbon transformation test as a positive control. Three replicates were used for all concentration in each test.

3.4.2 Plant seedling emergence and growth test

H. annuus and L. multiflorum

The plant seedling emergence and growth tests were conducted according to OECD guideline no. 208 (OECD 2000a). In briefs, seeds of the two plants *H. annuus* and *L. multiflorum* were soaked in tap water for 12 hours before planting into soil. Two and four seeds of *H. annuus* and *L. multiflorum*, respectively, were planted per pot containing soil spiked with the water (control), 2444 and 6110µmol/L of the reference compound boric acid, and 5 concentrations of the two tests compounds, all exposures conducted in triplicate.

An initial range finding test (DMNA: 1.11µmol/kg, 11.1µmol/kg, 111µmol/kg, 1110µmol/kg, 11100µmol/kg dry soil; 2NAE: 0.942, 9.42, 94.2, 943, and 9427µmol/kg dry soil) were conducted to identify the concentration-response area. Finally, with the same procedure as in the range finding test, the final test was conducted with concentrations of 11100µmol/kg dry soil using 6 replicates for DMNA and 15µmol/kg, 29µmol/kg, 59µmol/kg, 118µmol/kg, 237µmol/kg, 471µmol/kg, 943µmol/kg with triplicates of each for 2NAE.

After proper planting of the seeds, plants were fertilized every 3 days for the entire experiment. The growth conditions included 16 hours light per day at 80 µmol/m^2/sec and a constant temperature of 16 °C. The data were collected by weighing the biomass of the seedlings after 28 days and expressing the results as wet weight.

3.4.3 Plant vegetative vigour test

H. annus and L. multiflorum

Seeds of *H. annuus and L. multiflorum* were soaked in tap water for 12 hours before sowing in soil. Two and four seeds of *H. annuus* and *L. multiflorum* were planted per pot, respectively. After emergence of two to four leaves (OECD 2006b), the test chemicals and water (control) were spread on the leaves as an aerosol.

Three replicates of 5 concentrations of 2NAE or DMNA, plus controls (negative and positive) were used for both plants. The concentrations of chemicals were 8.88, 44.4, 222, 1110, and 5550µmol/kg for DMNA and 7.54, 37.7, 118, 943, and 4713µmol/kg for 2NAE.

After foliar application of test chemicals, plants were fertilized and grown under the conditions described above. After 28 days, plants were weighed and wet weight determined.

3.4.4 Soil microbial test

N-transformation test

The OECD N transformation test(OECD 2000a) measuring nitrification as nitrate production in soil amended with finely ground nutrient rich plant material was carried out with 10 g soil placed in 120 ml bottles.

One negative control, one positive control and five concentrations for both amines with 3 replicate each were conducted. Distilled water as negative and cadmium sulphate was used as positive control at a final concentration of 0.65µmolCd/kg of dry soil.

Plant material added as substrate was taken from young (2 month) properly fertilized greenhouse grown clover, *Trifolium subterraneum,* dried and ground < 2mm and added at 5 g/kg (0.05g) per sample and finely mixed with a spatula.

Treatments with amines were amended with appropriate serial dilutions DMNA: 1.11, 11.1, 111, 1110, 11100µmol/kg dry soil; and 2NAE: 0.942, 9.42, 94.2, 943, and 9427µmol/kg dry soil. Bottles were then loosely sealed in order to permit air exchange under a humid atmosphere and incubated in the dark at 20°C for 12hours and 28 days. After 12 hours and 28 day, the amount of nitrate formed in each treated bottle and controls was determined according the OECD Guideline no.216 (OECD 2000a). In essence, nitrate was extracted from 10 g soil by shaking the samples with 50 ml 0.1MKCl solution at 150 rpm for 60 min (OECD 2000a). Extracts were then centrifuged and filtered, and the liquid phases were sent for NO_3 analysis at Eurofins (Moss, Norway).

C-transformation test

A standard C-transformation test (OECD 2000b) was prepared with a similar design to the N-transformation test above, using 10 g soil per bottle and the same amounts of 2NAE or DMNA, the same controls treatments and the same number of replicates. After adding the different concentrations of 2NAE or DMNA, 30 mg (3g/kg soil) of glucose were added to half of the bottles as a C source, and the bottles sealed with rubber septa and crimp caps. The

remaining bottles were incubated in open bottles for 28 days prior to addition of glucose and capping, as described above

CO_2 production was measured after incubation for 12 hours at 20 °C by an Environmental Gas Analyzer for CO2 (EGM-4, http://www.ppsystems.com/co2_gas_analyzers.htm). Sampling of headspace gas was achieved by connecting the IR cell and gas circulation pump to the bottle head space by inserting two needles connected with tubes in a circular loop. The CO_2 concentrations were recorded and corrected for ambient CO_2 in the tubes.

4. Statistical treatment

Toxicity was expressed as inhibition of growth and at the given concentration and the effective concentration causing 50% effect (EC_{50}) were calculated using EPA Probit Analysis, Version 1.5 (http://www.epa.gov/nerleerd/stat2.htm), Toxicity responses obtained from different concentration were compared with control by one-way analysis of variance (ANOVA), at 95 % significant level ($p<0.05$) using the statistical program software R (http://www.r-project.org) to determine the No observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). Statistical significant differences between different concentrations were determined by the post hoc test *"Turkey HSD"* using the statistical program R (http://www.r-project.org).

5. Result

Toxicity results of the two transformation products of amine are presented below assessed in the battery of tests using two plant species and a soil microbial community.

5.1.1 Effects of 2NAE on plant seedling emergence and growth after soil exposure

Seedling emergence and growth test was conducted on *L. multiflorum and H. annuus* by exposing to 2NAE using concentrations over a log10 interval. The test were then repeated with a more narrow concentration range to achieve good quality concentration-response curves (CRCs) to derive NOEC, LOEC and EC_{50} values.

Range finding test

Effects of the reference compound boric acid and 2NAE on seedling emergence and growth were determined after 28 days exposure of the two plant species, *L. multiflorum* and *H. annuus* (fig. 5).

Boric acid, which was used as positive control (reference compound), was toxic to both, *L. multiflorum* and *H. annuus* at 2444µmol/kg after exposed for 28 days. Seedling emergence and growth were inhibited by a factor of approximately two compared to the control. Concentrations of 0.1 to 94µmol/kg 2NAE were non-toxic to the two plant species during their growing stage. Seedling emergence was completely inhibited by 2NAE at concentrations of ≥ 942µmol/kg (100mg/kg) in both species. The toxicity of both boric acid and 2NAE were similar in the two species tested, although *H. annuus* achieved about 2 times higher weight during the 28 days growth period.

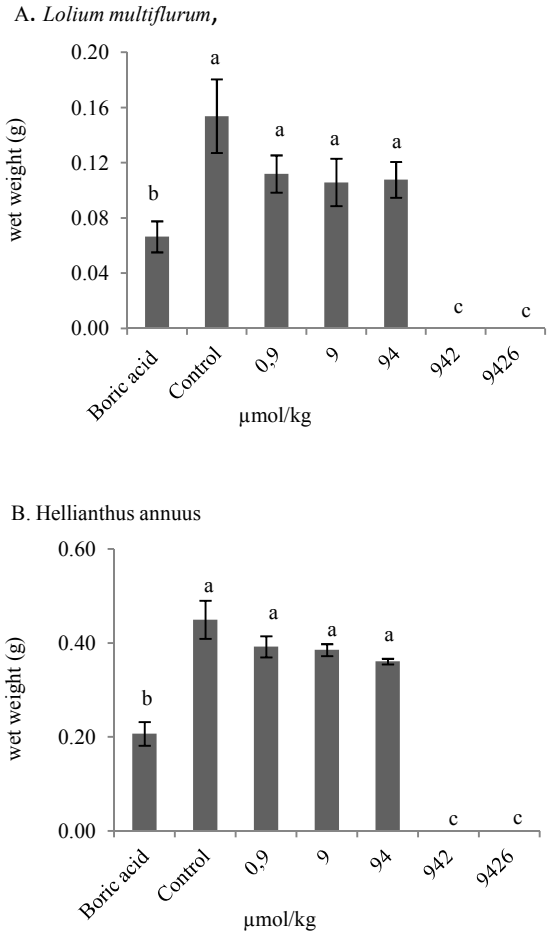


Figure 5.Seedling emergence and growth in Lolium multiflorum (A) and Helianthus annuus (B) after 28 days exposure to water (control), 2444µmol/kg Boric acid (reference compound) and different concentrations of 2-(nitroamino) ethanol. Data depict the mean ± SD (n=3) and the letters (a-c) denote groups that are statistically different (P<0.05).

Final test

A final test was conducted to determine the EC_{50}, LOEC and NOEC for the two plant species (fig. 6). As seen for the range finding test, boric acid was toxic at a concentration of 2444µmol/kg leading to a plant weight of less than half of that of the control group (45% decrease of biomass on *L. multiflorum* and 35% on *H. annuus*). Unlike the range finding test, the final test yielded high-quality CRCs for 2NAE in the concentration range of 30-1000 µmol/kg for the two species. At concentration of 118µmol/kg, the weight of *L. multiflorum*

was about 50% of the control, whereas at 471µmol/kg 95% growth of *L. multiflorum* inhibited. In a similar pattern, growth of *H. annuus* was reduced by 50% at 118 µmol/kg and complete growth inhibition was observed at 942µmol/kg. In both plant species, < 60µmol/kg concentrations no significant effects were observed. A summary of the NOEC, LOEC and EC_{50} for *L. multiflorum* and *H. annuus* have been compiled in table 3.

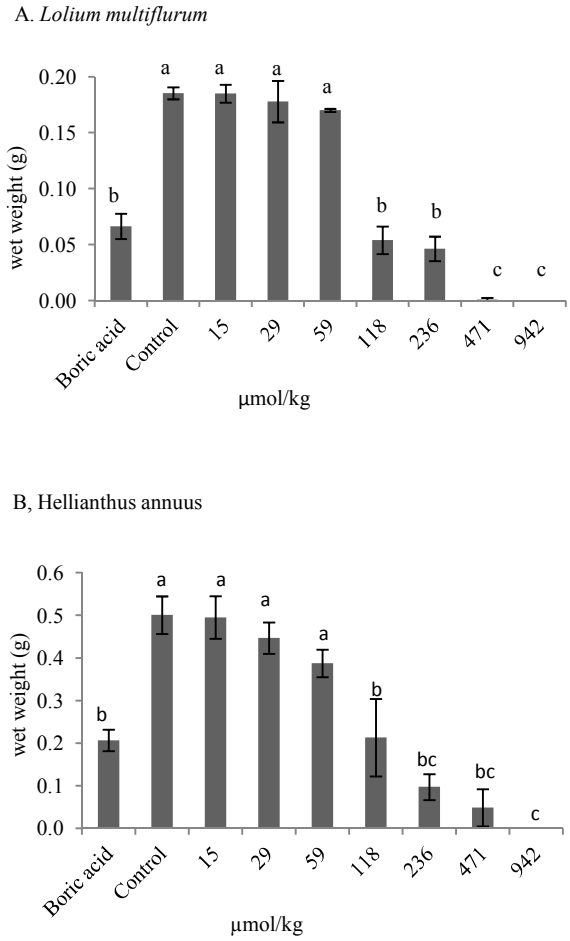


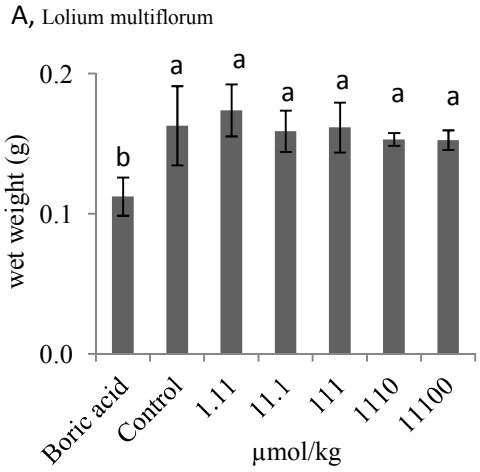
Figure 6.Seedling emergence and growth in Lolium multiflorum (A) and Helianthus annuus (B) after 28 days exposure to water (control), 2444µmol/kg Boric acid (reference compound) and different concentrations of 2-(nitroamino) ethanol. Data depict the mean±SD (n=3) and the letters (a-c) denote groups that are statistically different (P<0.05).

5.1.2 Effects of DMNA on plant seedling emergence and growth after soil exposure

A range finding test and a limit test was conducted for DMNA with the species *L.multiflorum* and *H. annuus*.

Range finding test

The range finding test was conducted to get the range of effective concentrations of DMNA. The result found in the range finding was not significantly different in both plants and it was not toxic at all. But boric acid as reference compound still had adverse effect which inhibited the growth of *L. multiflorum* (A) by half and *H.annuus* (B) by 35% comparing to the negative control group.



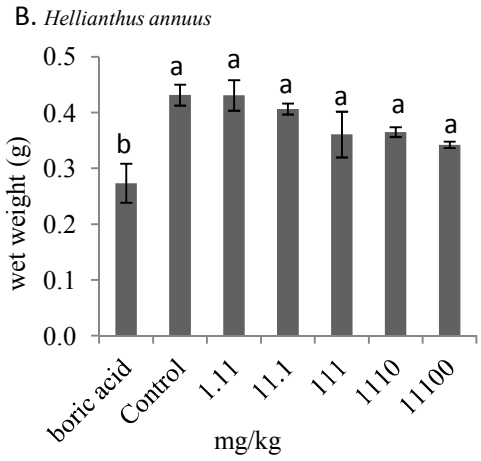


Figure7.Range finding test of seedling emergence and growth in Lolium multiflorum (A) and Helianthus annuus (B) after 28 days exposure to water (control), 2444µmol/kg Boric acid (reference compound) and different concentrations of 2-(nitroamino) ethanol. Data depict the mean ± SD (n=3) and the letters (a-b) denote groups that are statistically different (P<0.05).

Limits test

Limit test had carried out to check for any toxicity of DMNA again exposing to the highest concentration (11100) and using six replicates.

Growth decreased by less than 15% in both species was observed but it was not statistical different. In this test the adverse effect of boric acid was observed and it was little lower than 50% comparing to the negative control (water). The No Observed Effect Concentration and LOEC estimation is compiled in table 3.

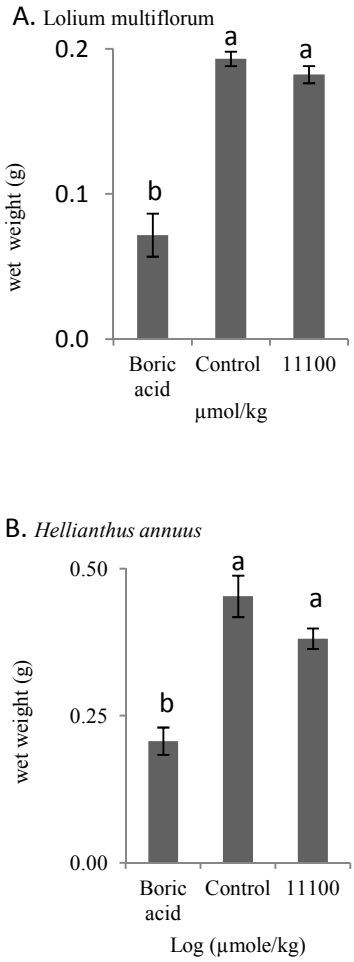


Figure8.Llimit test of seedling emergence and growth in Lolium multiflorum (A) and Helianthus annuus (B) after 28 days exposure to water (control), 2444µmol/kg Boric acid (reference compound) and different concentrations of dimethylnitramine. Data depict the mean±SD (n=6) and the letters (a-b) denote groups that are statistically different (P<0.05)

5.1.3 Effects of 2NAE on plant growth after foliar exposure

2-(nitroamino) ethanol was exposed sprayed on leaves to simulate direct impact of atmospheric exposure via precipitation and wind was conducted

The result surprisingly had positive effect. In this test boric acid decreased the growth of *L. multiflorum* by greater than 35% from the growth observed in the control group. The effect of

the concentrations (38µmol/kg and 119µmol/kg) was not significantly different from the control ($P < 0.05$). Growth increase on *L. multiflorum* started from 943µmol/kg and growth increase was by 22%. The highest concentration (4713µmol/kg) boosted the growth of *L. multiflorum,* It increased 38% of higher than the control group.

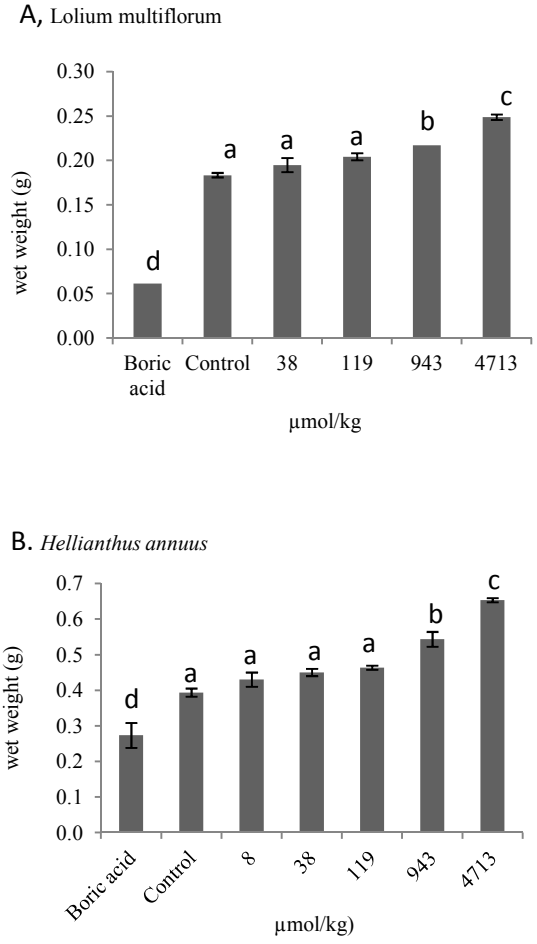


Figure9.Plant vegetative and vigour test using Lolium multiflorum (A) and Helianthus annuus (B) after 28 days exposure to water (control), 2444µmol/kg Boric acid (reference compound) and different concentrations of 2-(nitroamino) ethanol. Data depict the mean±SD (n=3) and the letters (a-d) denote groups that are statistically different (P<0.05)

The pattern of the effect induced by 2NAE on *H. annuus* was almost similar to *L.multiflorum* except the adverse effect of growth decrease observed by boric acid on *H. annuus* was half of the decreased growth observed on *L.multiflorum*. The similar 2NAE concentrations (943 and 4713µmol/kg) which induced significant growth increase on *L. multiflorum* were also found to induce significant growth increase on *H. annuus*. But at this time the growth increase at the concentrations (943 and 4713µmol/kg) of 2NAE were two times greater than the growth increased by the same concentrations on *L. multiflorum*.

5.1.4 Effects of DMNA on plant growth after foliar exposure

Dimethylnitramine was exposed sprayed on leaves to simulate direct impact of atmospheric exposure via precipitation and wind, likewise 2NAE.

The first two concentrations of DMNA effect on *L. multiflorum* were not significantly different from the negative control. But boric acid, the positive control induced 35% growth decrease. Unlike the boric acid adverse effect the last two concentrations of DMNA (1110 and 11100µmol/kg) imposed significant growth increase by 21 and 37% of the negative control (water) respectively. The No observed effect concentration and LOEC of DMNA on *L. multiflorum* refer to table 3.

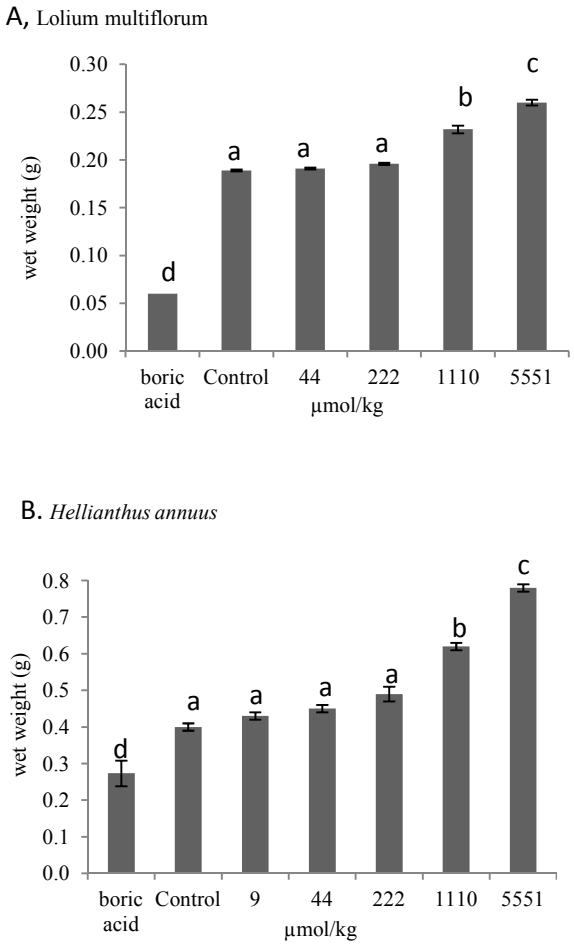


Figure 10. Plant vegetative and vigour test using Lolium multiflorum (A) and Helianthus annuus (B) after 28 days exposure to water (control), 2444 µmol/kg *Boric acid (reference compound) and different concentrations of dimethylnitramine . Data depict the mean±SD (n=3) and the letters (a-d) denote groups that are statistically different (P<0.05)*

The effect of DMNA on *H. annuus* started with one step lower concentration (222µmol/kg) and also the magnitude of growth increased was twice of the growth increased observed *on L. multiflorum*. The growth increase at the concentrations of 222 and 1110µmol/kg were nearly half of the control group. But at the highest concentration (11100µmol/kg) the growth of *H. annuus* boosted almost by double of the growth observed with the control group which was

90% increase of growth. Again refer to table 4 for the NOEC and LOEC of DMNA *on H. annuus.*

5.2.1 Effects of 2NAE and DMNA on soil microbial nitrogen transformation

Nitrogen transformation test was conducted to assess the effect of 2NAE and DMNA on soil organisms relating to their potential metabolic activity in converting organic matter to NO_3.

Generally the effect of 2NAE on the nitrogen transformation test was positive. Cadmium decreased the NO_3 formation by 80% from the control. 2-(nitroamino) ethanol induced NO_3 formation progressively more following the concentration increase. The effect of 2NAE on the nitrogen transformation was not significantly different from the control at concentrations from 1.1 to 111µmol/kg. Outside of the trend, the second concentration (11.1µmol/kg) increased the amount of NO_3 by 25% unlike the first (1.11µmol/kg) and the third (111µmol/kg) concentrations. The highest concentration of 2NAE increased the amount of NO_3 three times higher than the NO_3 formed by the control. The No Observed Effect Concentration and LOEC of 2NAE on this test are compiled in table 3.

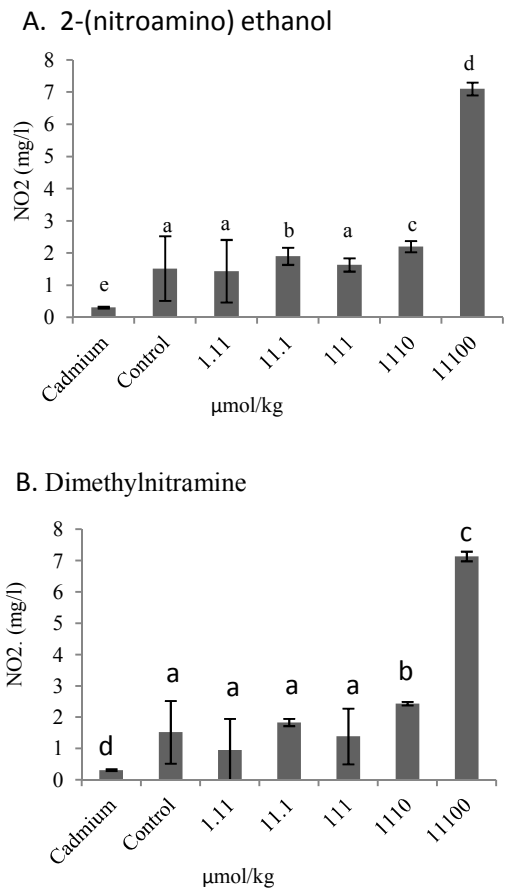


Figure11. Soil microbial nitrogen transformation on microbial measured after 28 days exposure to water (control), 0.65µmol/kg cadmium sulphate and different concentrations of 2-(nitroamino) ethanol and dimethylnitramine. Data depict the mean ±SD (n=3) and the letters (a-e) in chart (A) and the letters (a-d) in chart (B) denote groups that are significantly different (P<0.05).

Dimethylnitramine (figure 11B) also like 2NAE promoted the nitrogen transformation activity of the microorganism unlike the strong inhibition of cadmium. The inhibition of cadmium on microbial decreased the amount of NO_3 formation by 80% from the control. The significant increase of NO_3 by DMNA started at concentration of 1110µmol/kg. It was 60% higher than the NO_3 formed by the control. The highest concentration (1110µmol/kg) of DMNA again induced three times higher than that of NO_3 formed by control sample. Again the No Observed Effect Concentration and LOEC of DMNA are compiled in table 3.

After seeing the induction of NO_3 in the nitrogen transformation test we run additional experiment with same procedure without adding organic substrate and the NO_3 formed were 3.5 ± 0.3 mg/kg from 2NAE and 5.2 ± 0.3 mg/kg from DMN.

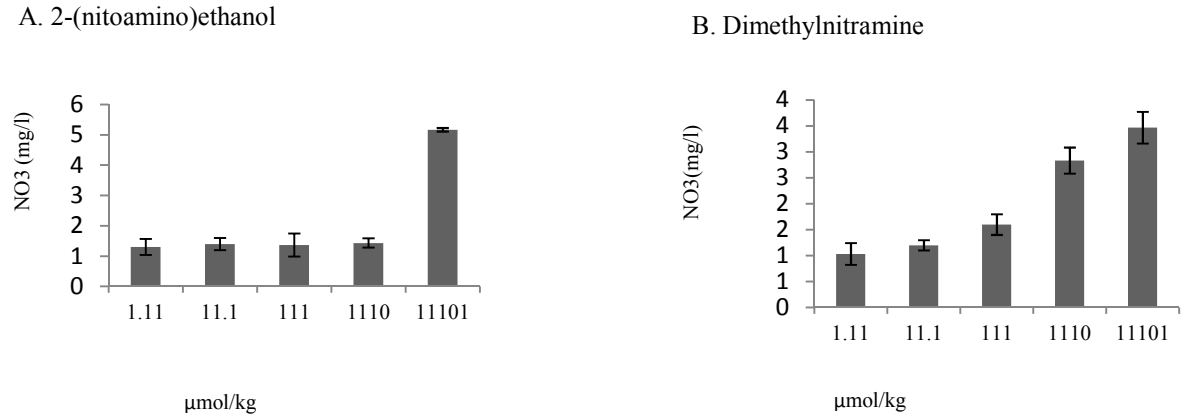


Figure12. Soil microbial nitrogen transformation measured after 28 days exposure to different concentrations of 2-(nitr amino) ethanol and dimethylnitramine conducted without adding organic substrate (Clover) to see the NO_3 formed either to be from the substrate only or also from the amines. Data depict the mean ±SD (n=3)

5.2.2. Effects of 2NAE and DMNA on soil microbial carbon transformation

The carbon transformation test conducted to support the nitrogen transformation test, because the finding supported with both transformations makes it strong. The carbon transformation like the nitrogen transformation increased the carbon formed.

In this test (figure 11), the amount of carbon produced was very similar using both amines. The first three consecutive concentrations (1.11, 11.1, and 111µmol/kg,) were not significantly different from the control (negative control) with both, 2NAE and DMNA. The positive control (Cd), at 0.65µmol/kg (50mg/kg) induced 40% decrease of CO_2 than the CO_2

produced by the control group. The highest concentrations of 2NAE and DMNA (1110, and 11100µmol/kg) induced above 25% higher CO_2 production than the control.

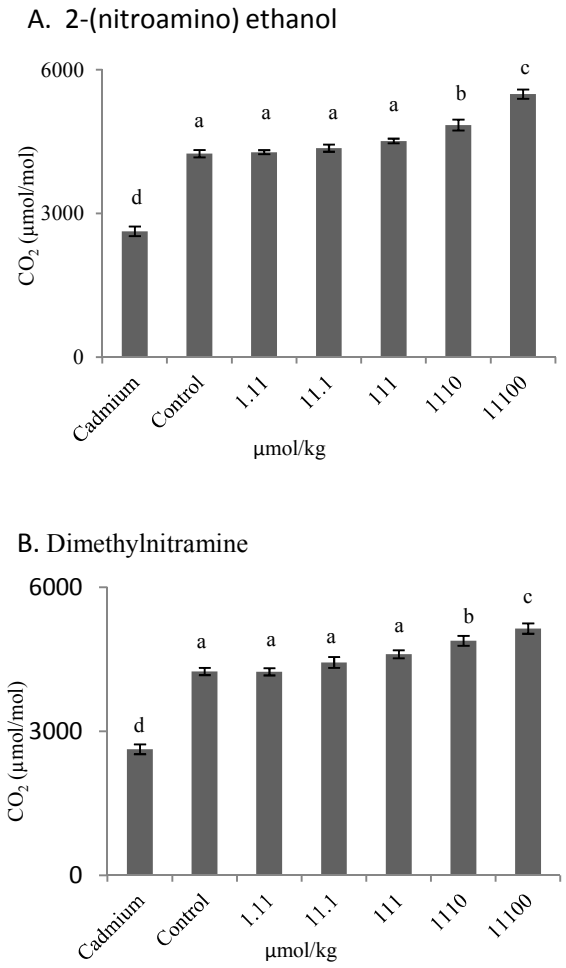


Figure 13.Soil microbial carbon transformation measured after 28 days exposure to water (control), 0.65µmol/kg cadmium sulphate and different concentrations of 2-(nitroamino) ethanol and dimethylnitramine. Data depict the mean ±SD (n=3) and the letters (a-d) denote groups that are significantly different (P<0.05).

The No Observed Effect Concentration and LOEC by 2NAE on the carbon transformation test were the same and it is compiled in table 3.

5.3 Ecotoxicological parameters for use in predictive risk assessment

Relatively speaking between these two compound 2NAE found to be more toxic in the interval of concentration we used. 2-(nitroamino) ethanol had EC50 of 118µmol/kg (12.5mg/kg), while DMNA was without adverse effect. LOEC of DMNA was >11100µmol/kg (1000mg/kg) which is almost 100 fold. In all rest toxicity tests, the stimulation of growth of 2NAE was started at smaller concentration than DMNA. The Lowest Observed effective Concentration of 2NAE in plant vegetative and vigour test was 15% greater than the LOEC of DMNA in both plant species. In the microbial nitrogen and carbon transformation, the No Observed effective Concentration and LOEC of both compounds were equal. Overall, 2NAE was more toxic than DMNA in the plant seedling and growth and was also more effective in stimulating growth on both plants.

Again, in plant seedling emergence and growth, the inhibition of growth was sensitive to 2NAE than DMNA with EC_{50} of 118µmol/kg and NOEC of 59µmol/kg. Our endpoint had opposite direction (growth inhibition and stimulation). It is not consistence enough to compare endpoint sensitivity. But between the stimulation of plant growth and stimulation of carbon and nitrogen transformation, the vegetative and vigour test is more sensitive with 15% less LOEC.

Plant species, *L.multiflorum* and *H. annuus* had equal sensitivity for both compound with equal EC_{50} of 2NAE and equal NOEC and LOEC of DMNA in the seedling and growth toxicity test. Even in the stimulation of plant growth the NOEC and LOEC of both compounds towards both species were exactly the same.

Again it was difficult to compare exactly efficiency of the rout of exposure. Our endpoints were not in the same direction. Both rout of exposure (root and leaves) had negative and positive effects (growth inhibition and stimulation of growth) at LOEC of 118µmol/kg of 2NAE and at 1110µmol/kg of both compounds.

We had acute negative and acute positive effect at 118µmol/kg of 2NAE and at 1110µmol/kg of both compounds. But the microbial test as chronic had positive effect at 1110µmol/kg for both compound again. The difference and similarity of responses were not only due concentration increase but also exposure rout.

The approximated estimation of PNEC for 2NAE (NOEC of 2NAE÷assessment factor (1000)) was = $6.3\mu g/kg*10^{-3}$ or 6.3ng/kg. The approximate estimation of PEC for our individual compounds from literature was < 0.01 ng/kg, and as crud the PEC for nitramines was less than 0.2ng/kg. And our calculated result of PNEC was only for 2NAE (NOEC of 2NAE÷assessment factor (1000) = 6.3µg/kg). Therefore through rough estimation our calculated PNEC was 5 times greater than the crud PEC or for rough estimation the PEC/PNEC ratio was 1/5 which is far less than 1.

Table 3 summary of NOEC, LOEC, and EC_{50} of 2NAE and DMNA of all tests

Name of the test	**Tested chemical**	**Test organism**	**NOEC** µmol/kg	**LOEC** µmol/kg	**EC_{50}** µmol/kg	**Remarks**
Seedling emergency and growth	2NAE	*H. annuus*	59	118	118	Induced inhibition
		L. multiflorum	59	118	118	
	DMNA	*H. annuus*	11100	>11100	N/A	Induced inhibition but not significant
		L. multiflorum	11100	>11100	N/A	
Plants vegetative vigour test	2NAE	*H. annuus*	111	943	N/A	Induced growth
		L. multiflorum	111	943	N/A	
	DMNA	*H. annuus*	222	1110	N/A	
		L. multiflorum	222	1110	N/A	
Microbial N-transformation	2NAE	Soil microbial	111	1110	N/A	Stimulates nitrogen- transformation
	DMNA		111	1110	N/A	
Microbial C-transformation	2NAE	Soil microbial	111	1110	N/A	Stimulates carbon- transformation
	DMNA		111	1110	N/A	

6. Discussion

These battery of toxicity tests using plants and microbial conducted following the OECD guidelines for testing chemicals. Our selected plant species and soil microbial are best representative of the main soil living organism.

Organisms such as plants and microbial are crucial indicators of the wellbeing of terrestrial soils. Any change on physiological and chemical processes of plants due to external interference affects plant growth. And any change on the metabolic activity of microorganism by external interference also alters the nitrogen and carbon transformation activity inside the soil matrix. As a result a strong association is known to exist between the higher plants' root surfaces and nitrogen fixing heterotrophic bacteria.

Plants can supply carbon sources to microorganisms in the soil and through secretions from plant root surfaces and microorganisms fixes the nitrogen nutrient as extracellular products.

Therefore by studying the endpoints (growth increase and inhibition in plants and inhibition and stimulation of nitrogen and carbon transformations of microbial activity) and the exposure rout sensitivity, both organisms can be best biomarkers to indicate quality of soil. Moreover these endpoints in our test organisms are very helpful for risk assessment.

6.1 Plant toxicity tests

Plants are the primary producers in the ecosystem converting energy directly from sun and become source of organic carbon to the soil. Any kind of unsuitable conditions which can alter the plant cellular physiology through external adverse effects and cause abnormality by decreasing strength and growth of plants are plant toxicity.

6.1.1 Plant seedling emergence and growth test

Boric acid is a soluble compound with a low occupational hazard, yet persistent over time and readily absorbed and taken up by plant roots (Anaka et al. 2008; Sorensen et al. 1997). The used boric acid as reference toxicant/positive control in allow verification of performance and comparison between different bioassays and tests (Stephenson et al. 1997). Boric acid, with concentrations of 536 mg/kg (6550µmol/kg) typically causes 20% inhibition (IC_{20}) of plant growth in temperate soils (EnvironmentalCanada 2005a), which was approximately consistent with our findings in *H. annuus and L. multiflorum* (species) that at 2444µmol/kg of boric acid in our case decreased 35% . There is a slight percentage difference between the effect and concentration applied between the reference test result and ours. But the reason can be from different use of soil matrixes. In our study the soil was a modification between field soil and OECD soil as described in the method section but the

reference test was used field soil sample. Therefore the organic matter constituents between the soil matrixes of the two tests were different.

The toxicity of different chemicals in different plant species is different because they don't have similar sensitivity. However, the results we get in the reference experiment were in agreement with the results we have from the literatures. In our study, at a concentration of 2.4mmolB/kg (2444µmol/kg) of soil, it inhibits growth in both plants by 35% to 40% in seedling emergence and growth and vigour and vegetative growth. Our testing plant species (*L. multiflorum and H. annuus*) are representative of dicots and monocots of the angiosperms' respectively. Angiosperms (flowering plants) are the major plants in dominating terrestrial environment.

Amines have been found to increase slightly the ion leakage from the water plant dotted duckweed (*Landolita punctata*) at doses of 1-10mg/L, and the leakage was significant at 100 mg/L (Kogan et al. 2000). Also, from the data done on definitive growth tests (Eckert et al. 1988b; Stantec 2006) to assess the toxicity of the amines MEA and DEA to three plant species, Alfalfa (*Medicago sativa*), Barely (*Hordeum vulgare*), and northern wheatgrass (*Elmus lanceolatus*), the IC_{25} values for these three species ranged from 584mg/kg to 2250mg/kg (MEA) and 858mg/kg to 4028mg/kg (DEA). Our data of 2NAE using both species (L. multiflorum and H. annuus) were approximately five fold lower than these findings and suggest 2NAE have higher adverse effect than the adverse effect shown by MEA and DEA.

The mode of action of observed adverse effect of 2NAE on both plant species exposed through moisturized soil might be due to change of soil chemistry after their application on the growing medium (soil). According to the publications (Hsu & Ashmead 1984) application of more nitrogenous substance into soil might rises the level of pH above 7.0, and an increase of pH above 7 creates basic condition which has chelating effect. Most essential nutrient which are responsible for most of physiological process and building of nucleic acid such as boron, calcium, iron magnesium and manganese will be unavailable.

Dimethylnitramine and 2-NAE are aliphatic amines with only two carbons for they are therefore very soluble in water depending on pH because of their basic characteristics. Our soil matrix was with very low organic matter, neutral pH and good electro-conductivity (table. 2); therefore as a result, both present testing chemicals are assumed to be bioavailable to our testing plants and microbial exposed in the soil as soil percolates. Due to the high water solubility of testing chemicals and very low organic matter constituent of our soil matrix, these chemicals (2NAE and DMNA) were readily exposed to testing organisms. According to literature (reference) amines with less than 5 carbons including our compounds are known to be easily biodegradable ((Aarrestad & Gjershaug 2009; Knudsen et al. 2009). Moreover these amines are known to be good sources of nitrogen and have a greater possibility to form ammonium and nitrate in the presence of microorganism. Ammonium and NO_3 are good chelating agent which inhibit the essential nutrients (iron, magnesium, boron, and calcium) in soil from being absorbed by the roots of the plants.

Therefore the increase of nitrogenous substance in the soil affects the up-taking of essential nutrients by increasing the pH and creating suitable condition for chelating effect. The toxic effect of both amines on the seedling emergence and growth test of the present study,

speculates to be due to the increased soil pH. Our soil pH measurement was greater than 7.8 which agreed with the above idea.

6.1.2 Plant vegetative and vigour test

Unlike the effect in seedling emergence and growth test, our result in vigour and vegetative test (Figures 9 and 10) using both amines (2NAE and DMNA) induced biomass-growth on both plants with to the increases of concentration in parallel. The stimulation of growth on this test can be due to direct nitrogen fertilization from amines. The literatures (Bergmann & Eckert 1990; Bergmann et al. 1994; Hsu & Ashmead 1984; Kogan et al. 2000) showed the presence of amines in plant cells integrated into the phospholipids and amines to stabilize the bio-membranes of plant cells in unsuitable conditions. Also thy proved that leaves sprayed amines to promote protein synthesis, to stimulates and improves flowering and seedling growth and acts as a plant bio-regulator.

Again, relative to stimulation of growth, we got from the plant vegetative and vigour test, former study done using different plant species, exposed to 0.02mol/L, and 10mg MEA / pot sprayed on leaves found to promote growth of basal stem and 14% increased biomass and grain yielded (Bergmann & Eckert 1990). They suggested that this result might be due to higher content of nitrogen fertilization from MEA.

Other studies also proved amines to be source of nitrogen fertilizer and bioregulators sprayed directly on leaves like we did on the vegetative and vigour test. To give examples, an experiment done barley sprayed 0.01M (0.3-0.5mg MEA per plant increased plant drought tolerance, and increased yield of grain protein by increasing the amount of Lucien, isoleucine

and other amino acids (Bergmann & Eckert 1990). Another study done on barley by spraying 0.5mgMEA per plant also found to stimulate recovering of cell membrane and decreased oxidative stress caused by herbicide (Mascher et al. 2005a). Therefore, amines are used as source of nitrogen fertilizer for protein synthesizing in plants by directly in taking through leaves after spray application. Therefore this study application of amine to plant root inhibited plant growth but direct application through the rout of leaves promoted increase of biomass. Both ways of rout of exposures: in the soil by up taking of roots and leave up taking of amines from the air hade efficiently given opposite effects.

6.2 Microbial test

Microbial in the soil plays great roll in energy cycle by decomposing and transforming nutrient inside soil. The microbial activity of carbon and nitrogen transformation affects the ecosystem positively as described in the introduction and result sections. But if available excess organic substrates are readily presented in fertile soils excess mineralization of nutrients through biodegradation occurs. The mineralization of nutrients then can contribute in acidification and eutrophication of lakes and vegetation as negative effects of microorganisms. The same mineralization can also contribute for increased atmospheric carbon and nitrogen emissions which can end up in global warming. As we know one of the main contributors to atmospheric pollution as a source of emission is soil.

As the result of N-transformation assay (fig.11) indicates, both amines induced induction of NO_3 transformation significantly at concentrations above 100mg//kg. In addition to the N-transformation test result, the additional nitrogen transformation test which conducted without adding of substrate (figure 12) showed, almost equal formation of NO_3 from the

substrate free samples (figure 12). This might explain that the microbial community of our soil samples was using easily their carbon source from the amines before using the substrate because the biodegradability of the amines were quicker than the added plant substrate. Also, when we look to the result of C-transformation (Figure 13), our result showed the increase of the CO_2 formation with the increased of amine concentration (Figure 13), therefore according the test results of both nitrogen and carbon transformation tests; both amines (2NAE and DMNA) induced microbial or growth.

Studies which have been done on the toxicity effects of the amines on soil microbial processes are not satisfactory, but some studies have shown that amines biodegrades (by microorganisms) to ammonia, acetaldehyde, acetate, nitrite, nitrate and nitrogen gas under both aerobic and anaerobic conditions, by involving processes of nitrification and dinitrification (Ndegwa et al. 2004). Amines biodegrades more easily in non-polluted soils compared with heavily polluted soils. Bacterial degradation studies on MEA was performed with soils that were contaminated 10 years ago and on uncontaminated soils from the same site that had been spiked with 1320mg MEA/kg dry wt (Hawthorne et al. 2005). In this study, the polluted sites showed slow biodegradation while MEA was completely degraded within 3 days of incubation at the spiked soil. In contaminated clay-rich soil, 2000 mg/kg of monethanolamine degraded by microbial under aerobic and anaerobic condition within 10 and 12 days respectively(Ndegwa et al. 2004).

Also 31000mg/kg of MEA also found to be biodegraded actively (Mrklas et al. 2004). Our data also showed that the foliar sprayed amines increased growth and from the data of nitrogen transformation (figure 11) we had induction of microbial growth. The analysis of

samples from the nitrogen transformation we conducted without the application of clover as source of nitrogen also confirmed that there was NO_2 formation (figure 12). These points cumulatively can lead us to speculate the amines itself to be source of nitrogen even before the microorganisms used the source of carbon from the substrate.

Finally concerning the environmental relevance to compare the ratio between what exposures is expected on modelled concentration relative to the result we get on the adverse effect, we did rough estimated calculation using crude PEC for nitramines; because we didn't get exact concentration estimation for our individual compounds. But even using the Crude PEC the ratio (PEC/PNEC) was 1:5 which is far less than one.

7. Conclusion

We had relative toxicity effect of 2NAE on the acute plant test. Both compounds triggered growth stimulation on the vegetative plant test exposed through roots. Both compound again stimulated microbial activity. The different responses, inhibition and stimulation were due to different characteristics of rout of exposure. Sensitivity of our plant species to both compounds was similar. Both compound stimulated the microbial activities. Additionally the rough estimation of risk quotient was 1:5 (0.2). Therefore both compounds are not risky.

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9. Appendixes of raw data

2NAE seedling emergence and vegetative growth Range finding test using *L. multiflorum*

	Boric acid						
	2.4		0,9	9	94	942	9426
Replicates	mmol/kg	Control	µmol/kg	µmol/kg	µmol/kg	µmol/kg	µmol/kg
r1	0.08	0.149	0.105	0.107	0.107	0	0
r2	0.07	0.183	0.128	0.122	0.095	0	0
r3	0.06	0.130	0.104	0.088	0.121	0	0
mean	0.07	0.154	0.112	0.106	0.108	0	0
STDEV	0.01	0.027	0.013	0.017	0.013	0	0

2NAE seedling emergence and vegetative growth Range finding test using *H. annuus*

	Boric acid						
Replicate	2.4		0,9	9	94	942	9426
s	mmol/kg	Control	µmol/kg	µmol/kg	µmol/kg	µmol/kg	µmol/kg
r1	0.23	0.491	0.367	0.390	0.367	0.000	0.000
r2	0.18	0.410	0.408	0.395	0.355	0.000	0.000
r3	0.21	0.448	0.402	0.371	0.360	0.000	0.000
mean	0.21	0.450	0.392	0.385	0.361	0.000	0.000
STDEV	0.025	0.041	0.022	0.013	0.006	0.000	0.000

2NAE Seedling emergence and vegetative growth final test using *L. multiflorum*

Replicates	Boric acid 2.4mmol/kg	Control	15 µmol/kg	29 µmol/kg	59 µmol/kg	118 µmol/kg	236 µmol/kg	471 µmol/kg	9427 µmol/kg
r1	0.08	0.189	0.186	0.188	0.171	0.062	0.055	0.003	0.000
r2	0.07	0.188	0.192	0.190	0.170	0.060	0.034	0.000	0.000
r3	0.06	0.179	0.176	0.156	0.169	0.040	0.050	0.000	0.000
Mean	0.07	0.185	0.185	0.178	0.170	0.054	0.046	0.001	0.000
STDEV	0.011	0.005	0.008	0.019	0.001	0.012	0.011	0.002	0.000

2NAE Seedling emergence and vegitative growth final test using H. annuus

Replicates	Boric acid 2.4 mmol/kg	Control	15 µmol/kg	29 µmol/kg	59 µmol/kg	118 µmol/kg	236 µmol/kg	471 µmol/kg	9427 µmol/kg
r1	0.23	0.533	0.508	0.467	0.395	0.264	0.071	0.060	0.000
r2	0.18	0.519	0.538	0.469	0.415	0.267	0.131	0.086	0.000
r3	0.21	0.451	0.440	0.404	0.352	0.108	0.089	0.000	0.000
Mean	0.21	0.501	0.495	0.447	0.387	0.213	0.097	0.048	0.000
STDEV	0.025	0.044	0.050	0.037	0.032	0.091	0.030	0.044	0.000

DMNA seedling emergence and vegetative growth limit test using *L. multiflorum*

Replicates	Boric acid 2.4 mmol/kg	Control	11 mmol/kg
r1	0.09	0.194	0.186
r2	0.07	0.195	0.190

r3	0.06	0.184	0.176
r4	0.08	0.195	0.179
r5	0.09	0.199	0.186
r6	0.06	0.193	0.176
Mean	0.07	0.193	0.182
STDEV	0.015	0.005	0.006

DMNA seedling emergence and vegetative growth limit test using *H. annuus*

	Boric acid 2.4		
Replicates	mmol/kg	Control	11mmol/kg
r1	0.23	0.423	0.365
r2	0.17	0.455	0.411
r3	0.21	0.516	0.369
r4	0.23	0.466	0.371
r5	0.19	0.425	0.392
r6	0.21	0.434	0.378
Mean	0.21	0.453	0.381
STDEV	0.023	0.035	0.017

DMNA vegetative vigour test using *L. multiflorum*

Replicate	boric acid2.4		44	222	1110	5551
s	mmol/kg	Control	µmol/kg	µmol/kg	µmol/kg	µmol/kg
r1	0.08	0.188	0.19	0.196	0.234	0.26
r2	0.06	0.189	0.192	0.197	0.227	0.263
r3	0.096	0.189	0.192	0.196	0.234	0.258

Mean	0.06	0.189	0.191	0.196	0.232	0.26
STDEV	0	0.001	0.001	0.001	0.004	0.003

DMNA vegetative vigour test using *L. multiflorum*

Replicates	boric acid 2.4mmol/kg	Control	9 µmol/kg	44 µmol/kg	222 µmol/kg	1110 µmol/kg	5551 µmol/kg
r1	0.31	0.39	0.43	0.44	0.48	0.61	0.78
r2	0.27	0.4	0.43	0.44	0.49	0.62	0.77
r3	0.24	0.41	0.44	0.46	0.51	0.63	0.78
Mean	0.27	0.4	0.43	0.45	0.49	0.62	0.78
STDEV	0.04	0.01	0.01	0.01	0.02	0.01	0.01

DMNA N-transformation test

Replicates	Cadmium 0.65µmol/kg	Control	1,11 µmol/kg	11,1 µmol/kg	111 µmol/kg	1110 µmol/kg	11101 µmol/kg
r1	0.33	0.36	0.38	1.70	0.37	2.50	7.10
r2	0.28	2.10	2.10	1.90	2.00	2.40	7.30
r3	0.33	2.10	0.37	1.90	1.80	2.40	7.00
mean	0.31	1.52	0.95	1.83	1.39	2.43	7.13
STDEV	0.03	1.00	1.00	0.12	0.89	0.06	0.15

Replicates	Cadmium 0.65µmol/kg	Control	1,11 µmol/kg	11,1 µmol/kg	111 µmol/kg	1110 µmol/kg	11101 µmol/kg
r1	0.33	0.36	0.32	1.60	1.40	2.30	7.30

r2	0.28	2.10	2.00	2.10	1.70	2.00	6.90
r3	0.32	2.10	2.00	2.00	1.80	2.30	7.10
mean	0.31	1.52	1.44	1.90	1.63	2.20	7.10
STDEV	0.03	1.00	0.97	0.26	0.21	0.17	0.20

N-transformation test result without substrate exposed to DMNA

DMNA	1,11	11,1	111	1110	11101
r1	1,6	1,2	1,1	1,3	5,1
r2	1,2	1,6	1,2	1,6	5,2
r3	1,1	1,4	1,8	1,4	5,2
mean	1,3	1,4	1,4	1,4	5,2
stdev	0,3	0,2	0,4	0,2	0,1

N-transformation test result without substrate exposed to 2NAE

2NAE	1,11	11,1	111	1110	11101
r1	0,8	1,3	1,8	2,8	3,2
r2	1,2	1,2	1,6	3,1	3,8
r3	1,1	1,1	1,4	2,6	3,4
mean	1,0	1,2	1,6	2,8	3,5
stdev	0,2	0,1	0,2	0,3	0,3

DMNA C-transformation using microbial

	Cadmium		1,11	11,1	111	1110	11101
Concentration	0.65µmol/kg	Control	µmol/kg	µmol/kg	µmol/kg	µmol/kg	µmol/kg
r1	2634.00	4335.00	4267.00	4297.00	4458.00	4747.00	5482.00
r2	2723.00	4199.00	4248.00	4347.00	4532.00	4970.00	5398.00
r3	2523.00	4209.00	4326.00	4442.00	4549.00	4821.00	5590.00
mean	2626.67	4247.67	4280.33	4362.00	4513.00	4846.00	5490.00
STDEV	100.20	75.80	40.67	73.65	48.38	113.58	96.25

C-transformation using 2NAE

	Cadmium		1,11	11,1	111	1110	11101
Concentration	0.65µmol/kg	Control	µmol/kg	µmol/kg	µmol/kg	µmol/kg	µmol/kg
r1	2634.00	4335.00	4203.00	4320.00	4699.00	4784.00	5139.00
r2	2723.00	4199.00	4328.00	4545.00	4590.00	4890.00	5034.00
r3	2523.00	4209.00	4192.00	4440.00	4533.00	4991.00	5249.00
Mean	2626.67	4247.67	4241.00	4435.00	4607.33	4888.33	5140.67
STDEV	100.20	75.80	75.54	112.58	84.35	103.51	107.51