Evaluation of toxicity in nematodes exposed to nitrosamines and nitramines relevant for CO_2-capture technology.

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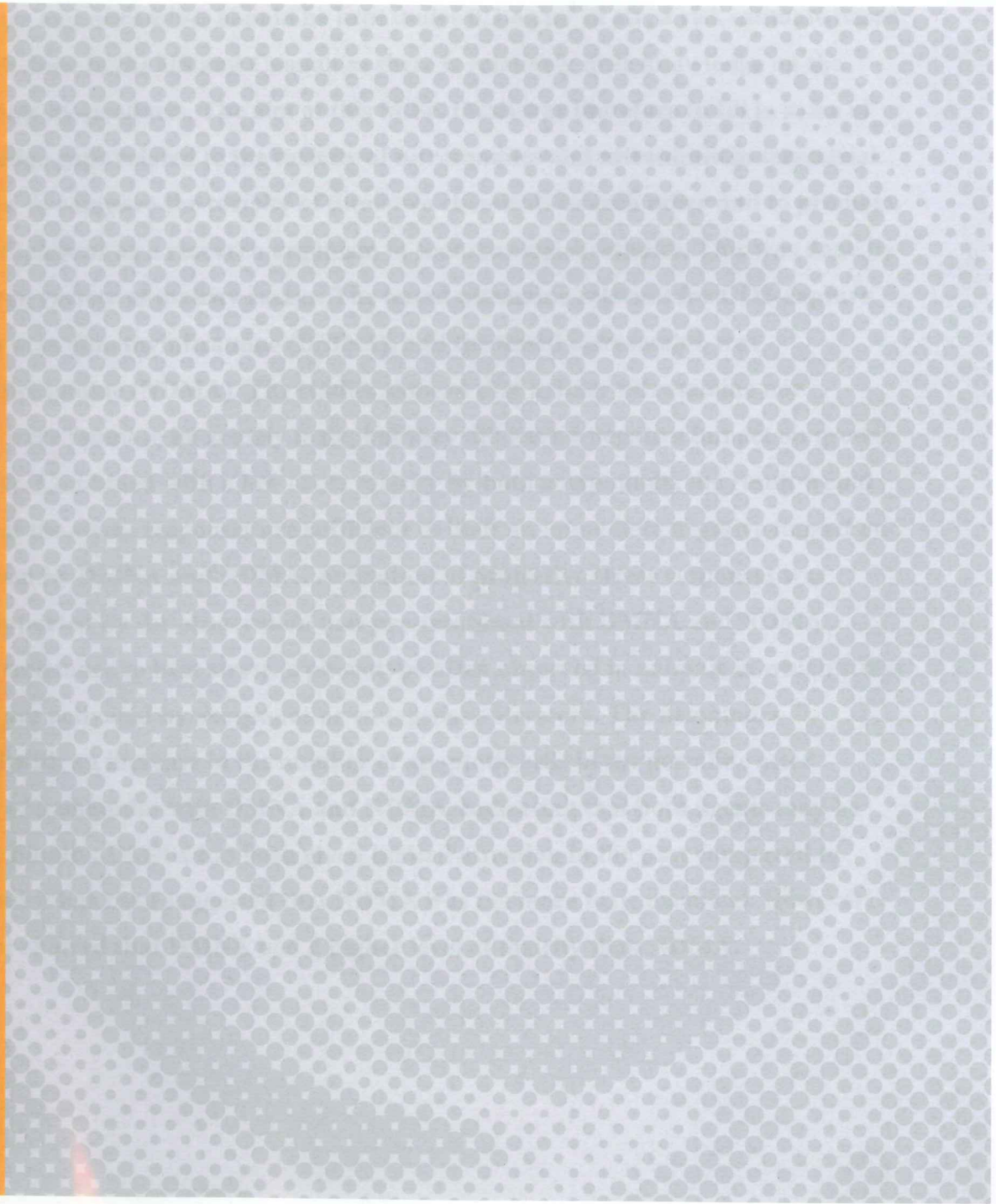

Evaluation of toxicity in nematodes exposed to nitrosamines and nitramines relevant for CO_2 capture technology.

Abstract.

Reduction of CO_2 emissions to slow down global warming is one of the main challenges of the industrialized world. One of the technologies available uses amines to capture the CO_2 in large combustion towers. The problem with this technical solution is that by-products generated from amine emission might have an impact on human health and the environment. The Norwegian Institute of Public Health advises that the exposure of nitrosamines and nitramines should be kept under 0.3 ng/m^3 in air, a risk estimate based on research done in the US and Canada as there is relatively little knowledge on the effect of these by-products. The aim of this M.Sc. thesis was therefore to fill in some of the knowledge gaps related to the toxicity of nitrosamines and nitramines in terrestrial vertebrates, to compare the toxicity of these two chemicals to each other and to promote the use of *C.elegans* as a research organism in ecotoxicological studies. This was done by using a large variety of toxicity assays. The tested nitramine relevant for CO2-capture technology was dimethylnitramine (DMNA), and the tested nitrosamine was dimethylnitrosamine (NDMA). The studies showed that neither nitrosamine nor nitramine were found to be acutely toxic, but they did have a negative impact on growth and fertility. Combined with a low acute toxicity by both compounds, the result would suggest that the stress responses are sufficient to limit an immediate negative impact on the organism by DMNA and NDMA. Activation of protective stress responses was further supported by the observation that pre-treatment with DMNA and NDMA increased resistance to heat stress, a phenotype often observed in animals in which stress responses are activated by exogenous or endogenous stressors. The nematodes responded with an increase in the number of apoptotic cells in the germline after treatment of nematodes with DMNA and NDMA, indicating that both agents may be genotoxic. Finally; DMNA has been given limited attention in the past compared to NDMA, and it has been suggested that NDMA was the more toxic chemical. However, the present showed that DMNA had a higher level of toxicity than NDMA.

Preface

This master thesis was a collaboration between the Norwegian University of Life Sciences (UMB), the Norwegian Institute of Water Research (NIVA) and the Biotechnology Centre, University of Oslo. The laboratory work was performed at the Nilsen group laboratory at the Biotechnology Centre, the analysis and thesis work was performed at NIVA.

Acknowledgements

First of all I would like to thank my main supervisor at UMB and NIVA, Professor II Knut Erik Tollefsen, for giving me the opportunity to work on this interesting and important project. I would also like to thank him for good help during the writing process, and for helping me interpret the data I got from my results.

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Thanks to Henok Kassahun for teaching me how to work with the nematodes, he is the most patient person I know. Thanks to Tanima Sengupta, Hanne Kim Skjeldam and Alfanso Schiavi for being excellent office-mates, I could always count on their support and advice. I would also like to thank Lene Alsøe for helping me with the molecular procedures in the lab. And of course I would like to thank the rest of the group; Kasia Arczewska, Laure Jobert, Eshrat Babaie and Marit Christine Bareid for a friendly and stimulating working environment.

I would also like to thank Ph.d. Deborah Oughton for being my co-supervisor at UMB, and Ph.d. Claire Coutris for discussion and cooperation of the practical lab work. It has been very educational working with them on the Exsira project.

Last but not least I would like to thank my family and friends for all their support, I could not have done this without their love and support.

Thanks for all your words of encouragement.

Table of contents

Abbreviations

BAC	Benzylcetyldimethylammonium chloride monohydrate
Biotek	Biotechnology Centre of Oslo
CO_2	Carbon Dioxide
CH	Methyl group
DIC	Differential Interference Contrast
DMNA	Dimethylnitramine
DNA	Deoxyribose Nucleic Acid
ENU	N-ethyl-N-nitrosurea
FHI	Norwegian Institute of Public Health
MEA	(2-nitroamino)- ethanol
mM	Milimolar
NDMA	Dimethylnitrosamine
NGM	Nematode Growth Medium
NH	Amine group
NILU	Norwegian Institute for Air Research
NINA	Norwegian Institute for Nature Research
NIVA	Norwegian Institute for Water Research
NO_3	Nitrate
OH·	Hydroxyl ion
O_3	Ozone

1. Introduction

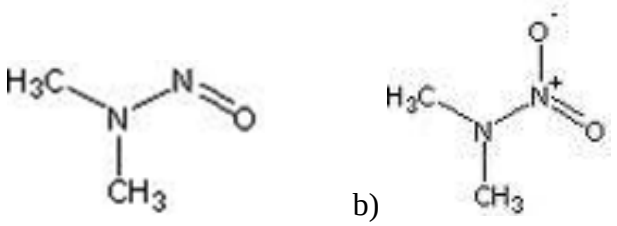
1.1. CO_2-capture technology using amines.

According to the Mongstad Technology Centre, the most promising technology today to capture CO_2 is by the use of amines. This is a technique using heated amines that bind CO_2 in a large combustion tower (Mongstad Technology Centre webpage, http://www.tcmda.com/no/Teknologi/Amin/). The CO_2 gas enters the bottom of the tower, where it comes in contact with liquid amines flowing downwards as the gas rises upwards. The gas then goes through a water wash to remove the amines, so that the clean gas can be released into the atmosphere. During the next step, the mixture of amines and CO_2 is transported to a regenerator where the amines are separated from the CO_2 by reversing the reaction between by steam. The CO_2 is then ready for storage. According to the Mongstad Technology Centre, 85 % of the CO_2 will be captured by using this method. However, there has been major concern about the possible health and environmental impacts of this technology since inevitably; there will be some amine emission. A project led by NILU, involving the Norwegian Institute for Public Health (FHI), the Norwegian Institute for Nature Research (NINA), the Norwegian Institute for Water Research (NIVA) and the University of Oslo Centre for Theoretical and Computational Chemistry (CTCC) have completed the first part of the project, a literature study concluding that more research is needed to fill in existing knowledge gaps (Knudsen *et al*, 1999). The problem seems not to be the amines themselves, but rather the degradation products that can be produced when the amines react with nitrous oxides present in the atmosphere. Nitrosamines are of a larger concern as several compounds of this class are reported to be carcinogens, and several nitramines are also suspected to be carcinogenic. The effects of amines may also have a severe effect on terrestrial organisms and fauna, and may possibly also lead to eutrophication of lakes (Knudsen *et al*, 1999).

1.2. Nitrosamines and nitramines.

Amines are derivatives of ammonia which have one or more hydrogen atom replaced by an alkyl group. They can be primary, secondary and tertiary, with one, two or three hydrogen atoms replaced by an alkyl-group, respectively. N-nitroso compounds are formed when secondary or tertiary amines react with a nitrosating reagent, whereas primary amines react with nitrosating agents to form unstable N-nitroso derivatives which are rapidly degraded to olefins and alcohols (Scanlan, 2003). Nitramines can be formed by nitration of amines (Selin, 2011). Many degradation products of amines relevant for CO2-capture technology have

caused concern, but the nitrosamine dimethylnitrosamine (NDMA, figure 1a) and nitramine dimethylnitramine (DMNA, figure 1b) are of highest concern due to their carcinogenic abilities.



a) b)

Figure 1 a) Dimethylnitrosamine (NDMA) and b) Dimethylnitramine (DMNA), which differs from NDMA by a hydroxyl-group. Pictures adapted from PubChem webpage: http://umbbd.msi.umn.edu/servlets/pageservlet?ptype=c&compID=c1199

1.2.1. Physico-chemical properties

Dimethylnitrosamine (NDMA, chemical formula $C_2H_6N_2O$) is one of the simplest nitrosamines, and soluble in most organic solvents (Heath & Magee, 1962). It is a yellowish, oily liquid with a characteristic odour. Its boiling point is 151-153 ^{0} C, its vapour density is 2.56 and the octanol/water partition coefficient is – 0.57. It is soluble in all common organic solvents and in lipids, and can be oxidized to nitramine or reduced to hydrazine or amine. It is also photochemically reactive, meaning it will degrade in sunlight (PubChem database, www.pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=6124#x27)

Dimethylnitramine (DMNA, chemical formula $C_2H_6N_2O_2$) is not as well documented as NDMA, but it is expected that is has similar chemical properties. It is a solid, crystalline chemical that is stable under normal conditions. Its boiling point is 187 ^{0} C and its density is 1.104 g/cm^3 (www.lookchem.com/Dimethylnitramine).

1.2.2. Atmospheric reactions.

Although most of the amines generated from current CO_2- capture technology are recycled in the combustion towers, a proportion of the amines will be emitted to air. The amines undergo physical and chemical reactions such as absorption, adsorption and photolysis, and many degradation products are formed (Shao & Stangeland, 2009). In general, amines are degraded by photolysis and by reaction with hydroxyl radicals during the day. The hydroxyl radical removes hydrogen from the methyl- and amino- groups generating amine radicals, which are then further degraded by ozone and nitrate (Shao & Stangeland, 2009).

An experiment done by Tuazon *et al* (1984) compared atmospheric reactions of and dimethylnitramine (DMNA) dimethylnitrosamine (NDMA) in an environmental chamber and showed that dimethylnitrosamine decays rapidly in sunlight.
Their research concluded that release or formation of NDMA is probably not a problem unless released at night. However, the photolysis of DMNA happens at a much slower rate and is of larger concern as it persists in the atmosphere for several days (Tuazon *et al*, 1984).

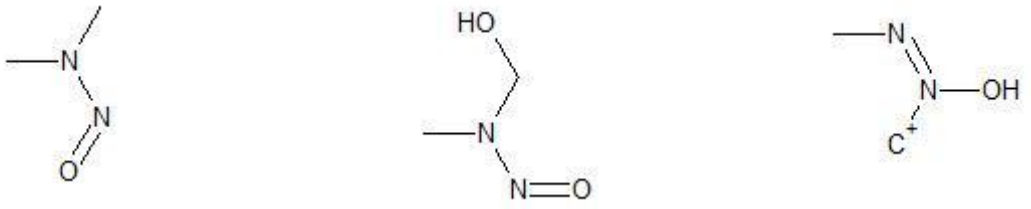
1.3. Ecotoxicology

The effect of the many degradation products of amines generated by CO_2 technology varies. The most described nitrosamine NDMA is not known to bioaccumulate, but the effects on plants are not extensively studied (Aarrestad & Gjershaug, 2009)
The literature on the effect of nitramines to flora and fauna is largely based on the use of explosives and is very limited (Aarrestad & Gjershaug, 2009).

1.3.1. Nitrosamines

Nitrosamines are a diverse group of secondary amines existing both naturally and as man-made chemicals, and are thoroughly studied. They exist as liquids, oils and volatile solids and are found in many everyday products such as cosmetics, tobacco and food (Låg *et al*, 2009). The acute effect of amines is liver necrosis, although lung lesions have also been reported (Heath & Magee, 1962). Nitrosamines are oxidised *in vitro* to powerful alkylating agents in the liver, and they also inhibit protein synthesis, alkylate liver protein and ribonucleic and deoxyribonucleic acids (Heath & Magee, 1962).

Dimethylnitrosamine (NDMA) is one of the simplest aliphatic nitrosamines, and has also been linked to the death of agricultural animals in Norway in the 1960's after they had been fed nitrite-preserved fish meal (Scanlan, 2003). NDMA is currently classified as a Cancer 2 carcinogen (Låg *et al*, 2009), and the Carcinogenic Potency Project database reports the TD50 (daily dose rate in mg/kg body weight per day) for NDMA to be 0.0959 mg/kg/day in rats (http://potency.berkely.edu).
NDMA is an indirect carcinogen, meaning it needs to be metabolically activated by cytochrome P450 oxidation to the carcinogenic methyl carbonium ion (figure 2) (Klaassen, 2008):



Dimethylnitrosamine (Procarcinogenic) → Hydroxymethyl (Proximate carcinogenic) → Methyl carbonium ion (Ultimate carcinogen)

Figure 2. The activation of NDMA into an ultimate carcinogen. (C.D.Klaassen, 2008)

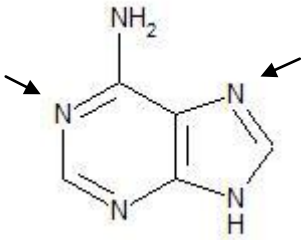
1.3.2 Nitramines

Compaired to the nitrosamines, the nitramines have received limited attention in the past decade (Klif final report, 2001). A study done by Malaveille *et al* (1983) concluded that nitramines require activation by hydroxylation and nitroreduction to become mutagenic. In their research they showed that bioactivation of dimethylnitramine (DMNA) in co-incubations with liver enzyme homogenates (S9-fractions) from rat enhanced the mutagenic rate in *Salmonella typhimurium* by a factor of 100. DMNA is also reported to be poisonous by intraperitoneal route and moderately toxic by ingestion (www.lookchem.com/Dimethylnitramine). The Carcinogenic Potency Project database reports the TD50 (daily dose rate in mg/kg body weight per day) for DMNA to be 0,547 mg/kg/day for rats (http://potency.berkely.edu).

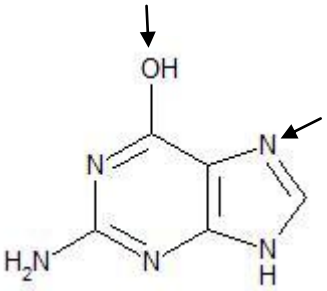
The carbonium ions of DMNA and NDMA are classified as alkylating agents, meaning they are toxic on the molecular level as they are able to react with DNA and form adducts, which might lead to mutations. Both nitrosamines and nitramines can cause alkylating DNA-damage by acting as strong electrophiles. Although the alkylation can happen at many sites of DNA (figure 3), some sites of DNA are more amenable to electrophilic attack due to a high negative electrostatic potential (Kondo *et al*, 2010). This makes the N^7 position of guanine the site being the most susceptible to alkylation; standing for 68 % of total alkylation after treatment with dimethylnitrosamine (Kondo *et al*, 2010). Although less common, the phosphate groups may also be alkylated (Klaassen, 2008). The common DNA adducts formed are O^6-

alkylguanine and O^4-alkylthymine, which one of these is the most carcinogenic is still debated (Klaassen, 2008). Treatment with alkylating agents may destabilize the glycosylic bonds resulting in formation of abasic sites in the DNA by depyrination and depyrimidation (Kondo *et al*, 2010). Figure 3 illustrates the most common sites where alkylation can take place.

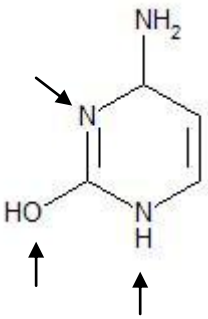
Adenine



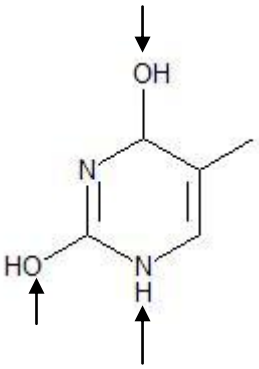
Guanine



Cytosine



Thymine



Phosphate

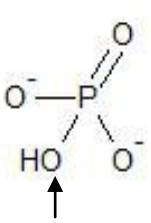


Figure 3. Examples of sites in the bases of nucleotides that can be alkylated and form adducts. Adapted from Klaasen, figures drawn in MedChem designer.

1.4 The nematode *Caenorhabditis elegans*.

Caenorhabditis elegans (C.elegans) is a common organism found in soil in most parts of the world (Wood, 1988). It is only about 1 mm in length and feeds mostly on bacteria. It is a common research organism used in both biomedical and ecotoxicological studies, and was the first multicellular organism to have its whole genome sequenced (Leung *et al*, 2008).

For research purposes, the worms are generally kept on Nematode Growth Medium (NGM) plates and fed with the *E.coli* strain OP50, a strain that has a limited growth as it is a uracil autotroph, and are normally studied under a stereomicroscope. Figure 4 demonstrates a normal view of a mixed plate with wild type (N2) *C.elegans* nematodes.

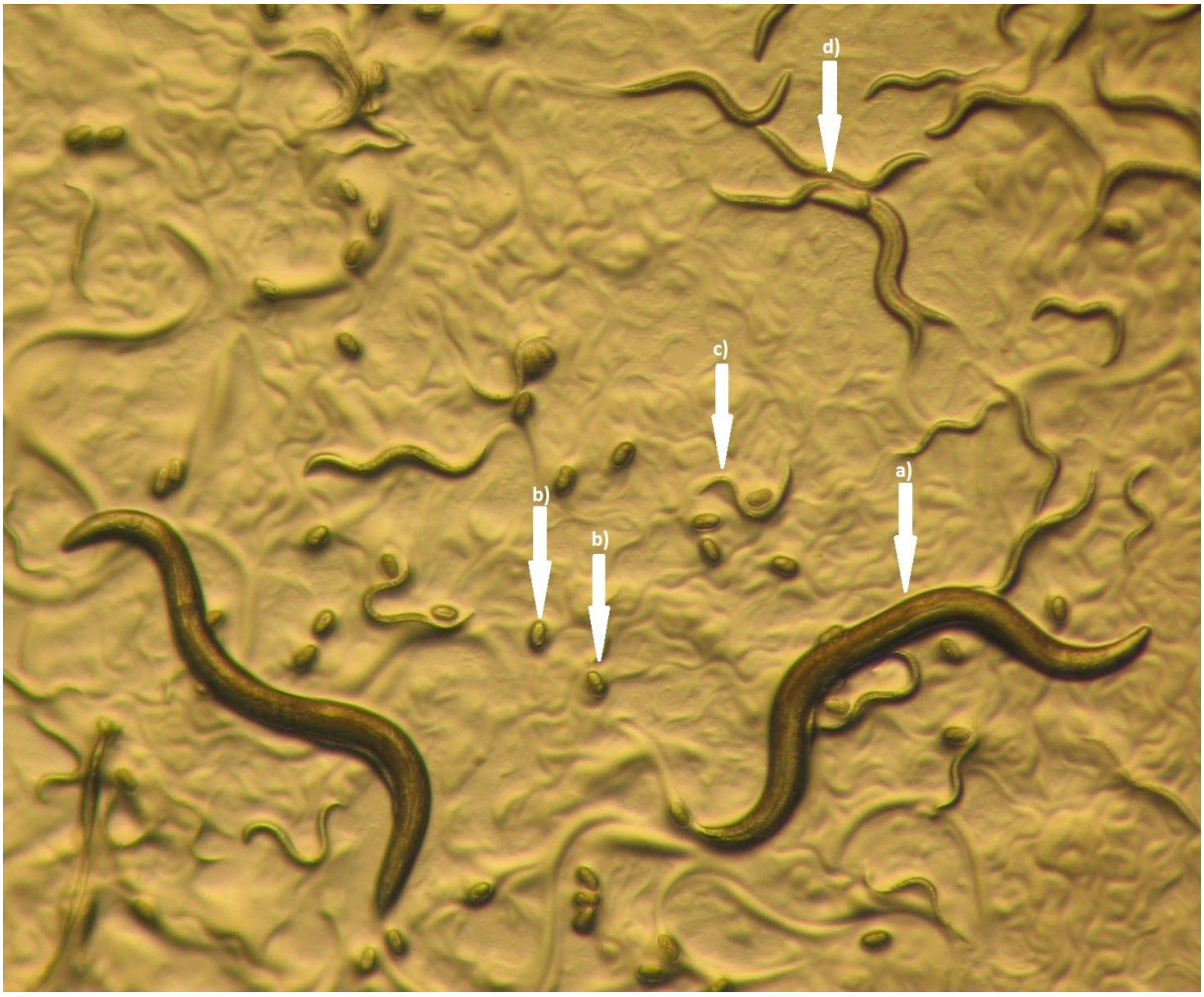


Figure 4. *A mixed plate of wild type Bristol N2 C.elegans seen through a stereomicroscope, 10X magnification. This picture shows: a) Adult hermaphrodite. b) Egg. c) L1 staged larvae. d) L2/L3 staged larvae. Photo courtesy of Anneke Maria Bruno.*

1.4.1. Life cycle of *C.elegans*

The life cycle of the *C.elegans* (figure 5) is short, taking only 3.5 days to reach adult stage at 20 ^{0} C. It is also possible to decrease the speed of the life cycle to six days at 15 ^{0} C, or increase the speed of the life cycle at 25 ^{0} C taking it 3 days (Hope 1999).

During the first larval stage, L1, the nematodes are about 250 µm long (Hope, 1999).

In this stage, many genes are switched on. Alternatively, if the environmental conditions are not favourable, the nematodes can enter a resting stage known as the *Dauer* stage instead of the L3 stage. Dauer larva are thinner than L3, and remain still most of the time unless provoked by touch (Hope, 1999). During the L2 stage there is rather little cell division, instead the existing cells are growing rapidly (Wood, 1988). In the L3 stage, hermaphrodites and males can be distinguished from each other. The L4 larva has a distinct white structure on its abdomen, which distinguishes it from the young adult and can be seen in a dissecting microscope.

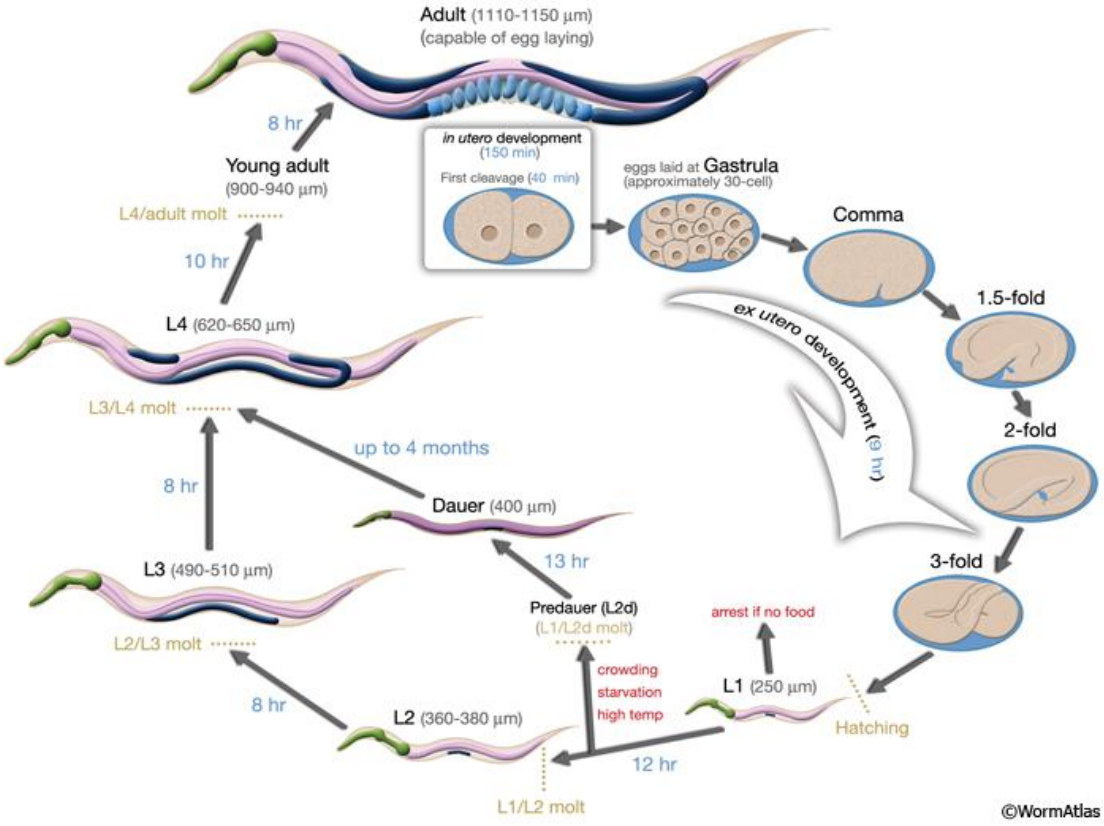


Figure 5. The life stages of the C.elegans, showing the development of the nematode C.elegans. Unfavourable conditions such as crowding, starvation and high temperature will lead them into Dauer stage, an alternative stage from L2. Adapted from www.wormatlas.org

1.4.2. Biology and anatomy of *C.elegans*

C.elegans is simple and small; the hermaphrodite consists of only 959 somatic cells, the male has 1031 (Wood, 1988). There are two sexes, hermaphrodites (XX) and males (XO) (Altun & Hall, 1999). The hermaphrodites produce both sperm and oocytes and can therefore reproduce by self-fertilization, but cannot fertilize each other. The males occur at a very low frequency of about 0.2 % (Altun & Hall, 1999). The males only produce sperm and are able to fertilize hermaphrodites, and can arise spontaneously or at increased rates under stressful conditions (Wood, 1988). This happens during the post-embryonic development and can be used as an endpoint in phenotypic assays (Emmons, 2005). Cross fertilization creates males and hermaphrodites in equal amounts, whereas self-fertilization only creates hermaphrodites with males occurring at the spontaneous, low frequency (Hope, 1999). The male can be distinguished from the female with its slightly smaller and thinner appearance. It also has a different structure at the posterior end (Hope, 1999).

Under stressful conditions the nematodes may go into Dauer stage, a state of hypometabolism which leads to a significant reduction in metabolism, an increased temperature and restricted oxygen supply (Lant & Storey, 2010).

Figure 6 gives an overview on *C.elegans* anatomy. It has a transparent structure, with a cylindrical body shape and tapered ends typical for nematodes (Altun & Hall, 2009). The epithelial system consists of a hypodermis that secretes cuticle which surrounds the worm (Altun & Hall, 2009). The nervous system is made up of ganglia in the head and tail, with the majority of nerve cells located around the pharynx (Altun & Hall, 2009) The nematodes respond to changes in chemical environment, mechanical stimuli, osmolarity and temperature, but the sensory tissue is also necessary for escape from Dauer stage, egg laying rate and feeding (Wood, 1988). *C.elegans* is a filter feeder, with a two lobed pharynx specialised in digesting the nutrients and pumping it into the gut (figure 7f) (Wood, 1988). The pharynx has its own nervous system, muscles and lumen (Altun & Hall, 2009). The reproductive system of the hermaphrodite has two bilaterally symmetric gonads. These are connected by a central uterus through the spermatheca (production and storage of sperm, and the area where the eggs become fertilised) (Altun & Hall, 2009). The germ cells travel through the gonads as they go through mitotic, meiotic and diakinesis (the final part of the meiosis) stages, in other words while undergoing maturation (Altun & Hall, 2009). In the loop of the gonad, the oocytes enlarge, detach from the syncytium and mature while moving to the proximal end (Altun &

Hall, 2009). The males are slightly smaller and thinner than the hermaphrodites, and have a distinctive tail. They start to develop male features in the L2 stage, with the most distinctive feature of the male being the tail (figure 7b), which works as a copulatory structure that can be seen in the stereomicroscope (Altun & Hall, 2009). The male has a single-armed gonad that opens to the exterior of the cloaca, at a modified epithelial chamber known as the proctodeum (male posterior gut) (Altun & Hall, 2009). The distinctive fan of the male tail includes rays, consisting of 18 sensilla, acting as sensory receptors. Upon copulation, the male locks on to the hermaphrodite with its two copulatory spicules into the vulva (Wood, 1988).

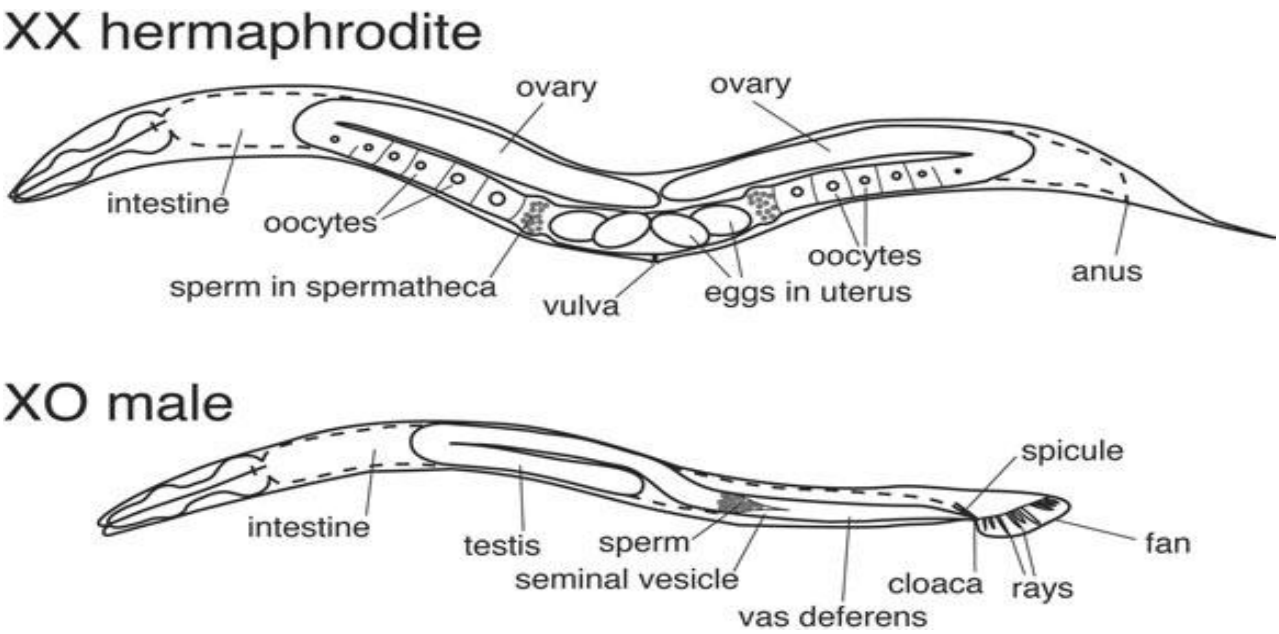


Figure 6. Showing the features of the hermaphrodite and the male C.elegans. The hermaphrodite has two bilobed gonads, whereas the male has a single lobed gonad. The male has a distinctive fan visually distinguishing him from the hemaprhodites. It is also slightly smaller and moves faster. Adapted from [www.wormbook.org.](http://www.wormbook.org)

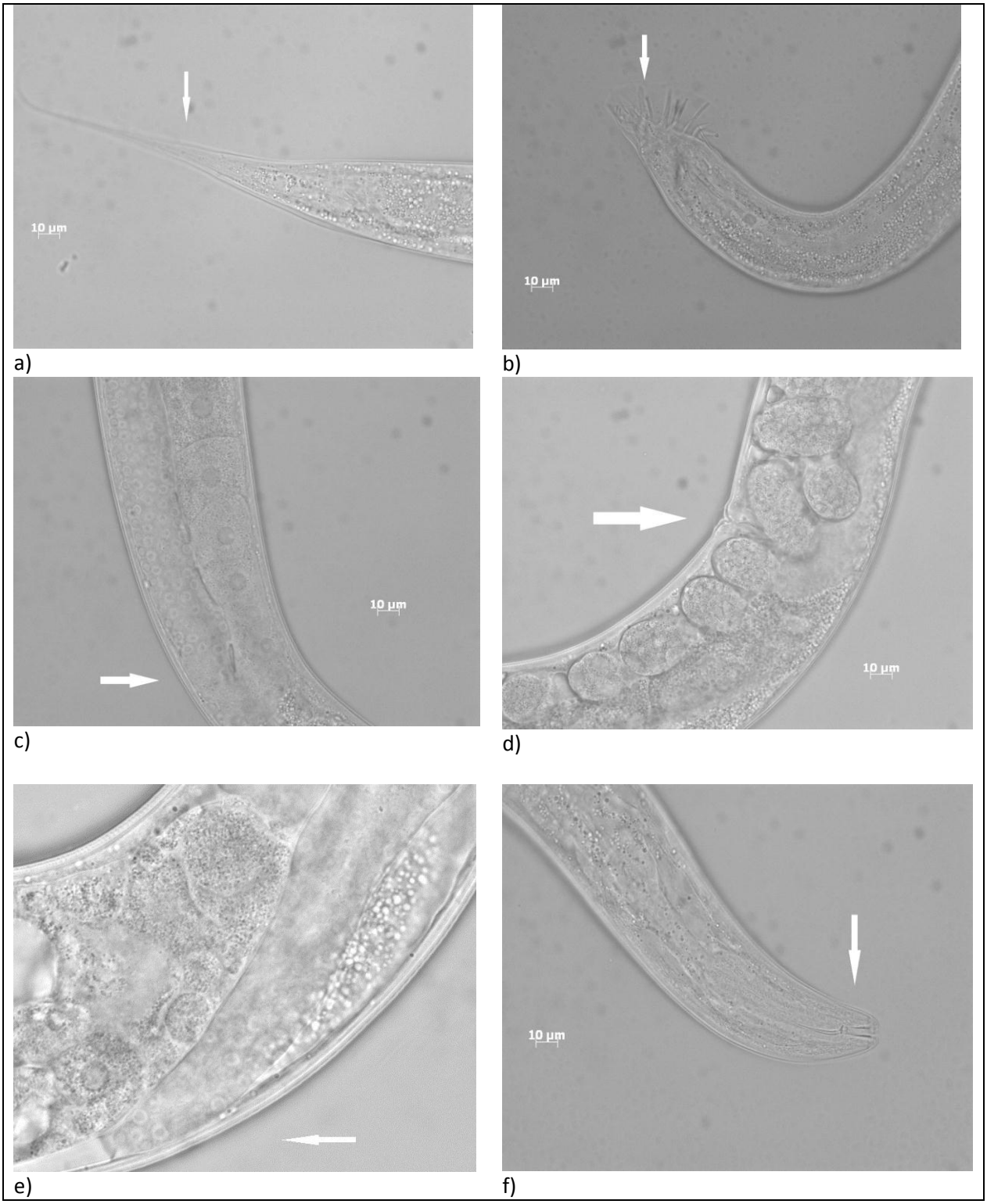


Figure 7 a) The hermaphrodite tail. b) The male tail. Increase in the number of males is a stress response measurable in toxicity assays. c) Maturing of oocytes. d) Embryos and the vulva. e) Mitotic cell region (lower left). f) The pharynx. Photos taken with AuxioVision stereomicroscope, 64 x magnification. Courtesy of Anneke Maria Bruno.

The genome of *C.elegans*

The genome of *C.elegans* consists of 1,00 x 10^8 base pairs according to the Wormbase WS226 release of 03.06.2011 (http://www.sanger.ac.uk/Projects/C_elegans/WORMBASE/DNA.shtml). This makes it a small genome (the size of the human genome is by comparison 3,00 x 10^9 base pairs). (Human National Human Genome Research Institute webpage). (http://www.genome.gov/11006943). The genome of *C.elegans* is spread over six haploid chromosomes in addition to the mitochondrial DNA (Wood, 1988). This set includes five autosomes and one sex chromosome (X) (Hodgkin, 2005). The sex chromosome determines the sex of the nematode; the hermaphrodites are diploid for all six chromosomes (XX), whereas the males are diploid for the autosomes but diploid for the sex chromosome (XO) (1988).

Germline development in *C.elegans*

The development of the germ cell line starts from a single primordial cell (P_4), which does not start to differentiate until the environmental conditions are favourable (Kimble & Crittenden, 2005). The cells grow exponentially during the larval stages L1 and L2, but arrest in stage 2 if the larvae go into Dauer stage (Kimble & Crittenden, 2005).
The germ line proliferation is controlled by the distal tip cells (DTCs), and they also maintain the germline mitoses in the adults.

Germline survival and apoptosis

There are two types of germline apoptosis; one is involved in germ cell death apoptosis in normal oogenesis, the other is DNA damage induced by environmental stressors which will be addressed later in this chapter (Gartner *et al*, 2008).

Figure 8 illustrates the physiological cell death of the germ cells in *C.elegans* initiated by the core apoptotic machinery necessary for apoptosis in *C.elegans*. The physiological germ cell apoptosis is CEP-1 independent, and the *cep-1* activates and starts working as a transcription factor in response to cytoplasmatic stresses (oxidative, heat, osmotic, starvation) and by DNA damage (Gartner *et al*, 2000). The apoptotic machinery consists of CED-3 (a human caspase homolog) and CED-4 (a human Apaf-1 homolog) (Gartner *et al*, 2000). The act of CEP-1 is to increase levels of CED-13 and EGL-1, which inhibit the CED-9 protein. The CED-9 protein in turn inhibits CED-4, which leads to activation of CED-3 (Gartner *et al*, 2000). Both

physiological and DNA damage germ cell apoptosis are suppressed by the apoptotic inhibitors PAX-2 and EGL-38 (Gartner *et al*, 2000).

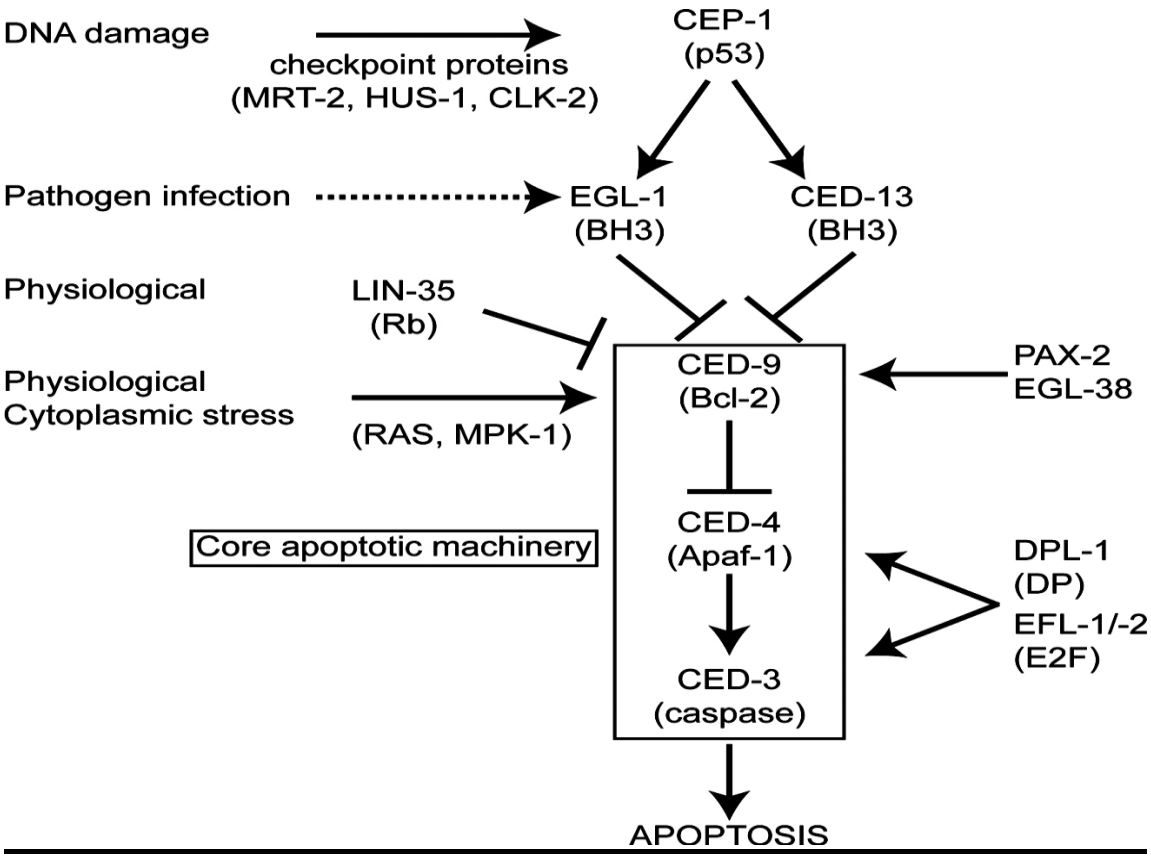


Figure 8. *Schematic overview of the germline apoptosis in C.elegans. The human gene equivalents are shown in brackets. Gartner et al, Germline survival and apoptosis, 2008. WormBook.*

Germline apoptosis in *C.elegans* is very suitable for identifying environmental stressors. Gartner et al (2008) studied the increase of apoptotic cells in the *C.elegans* germline (figure 9) exposed to N-nitroso-N-ethylurea (ENU) by using a transgenic *C.elegans* strain expressing the protein CED-1::GFP (Gartner *et al*, 2008). This protein highlights the somatic sheath around the dying cells, which can be observed in the EpiFluorescence microscope (figure 10 a and b).

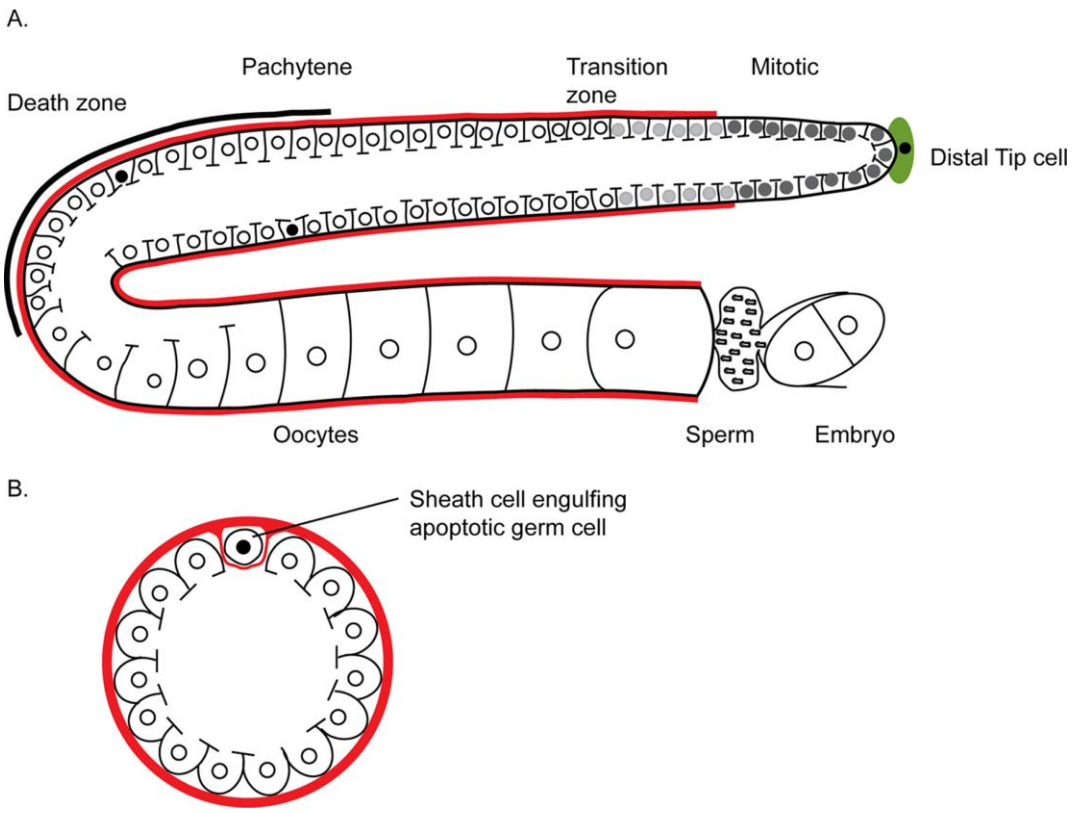


Figure 9. Schematic overview of the germline of C.elegans, showing the mitotic region on the upper right. The germ cells migrate towards the loop and mature into oocytes before they become fertilised and become an embryo. b) If the apoptosis mechanism is activated the germ cell becomes engulfed by a sheath cell. Gartner et al, 2008.

a) b)

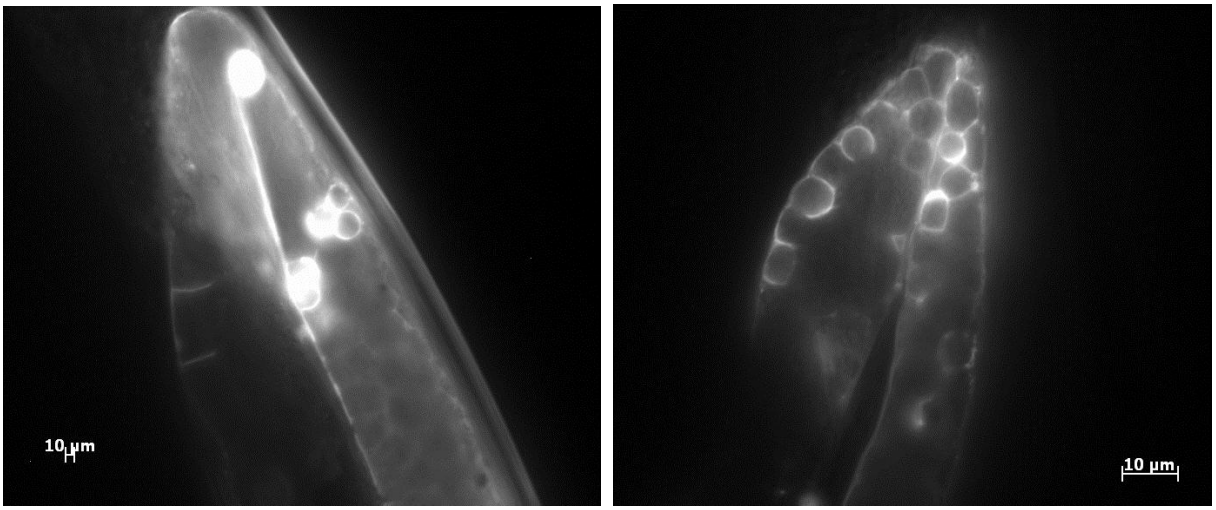


Figure 10. a) Control group shows few apoptotic corpses in the loop of the germline. b) Exposure to 5 mM ENU increases the number of apoptotic corpses, suggesting DNA damage. Pictures taken with AuxioVision with a fluorescent filter, 63x magnification, courtesy of Anneke Maria Bruno

1.4.3. *C.elegans* as an experimental organism

C.elegans has adapted to many different environments and can be found in soil all over the world, making it very relevant for ecotoxicological studies. It is an easy maintainable and low cost organism to work with, requiring only a petri dish with agar and *Escherichia coli* as a nutrient (Leung *et al*, 2008). It has a short life span, and produces about 300 offspring, allowing large quantities of worms to be obtained in a short period of time. Many physiological features observed in humans have also been observed in *C.elegans*, underlining its usefulness in neurotoxicology, genetic toxicology and environmental toxicology (Leung et al, 2008). It is also very useful for high throughput screening, and was the first organism to get its entire genome sequenced. About 60-80 % of all genes in humans are homologues of *C.elegans* genes (Leung *et al*, 2008). The possibility of monitoring whole populations *in vivo* increases the relevance for ecotoxicological studies. Various endpoints relevant in ecotoxicology can be studied, such as reproduction, brood size analysis and growth. The short life span allows long time studies over several generations taking much less time than traditional test organisms. As it was the first multicellular organism to have its whole genome sequenced, there is a large research network for *C.elegans* available online, such as WormBase, WormAtlas and WormBook to mention a few (Antoscheckin and Sternberg, 2007). The WormBase is a genomic database that provides the genomic sequences for C.elegans and closely related species, the WormAtlas is a webpage that provides all the specialized information of C.elegans anatomy, and the WormBook provides information on the biology of *C.elegans* as well as standard methods (Antoscheckin & Sternberg, 2007). In short, these webpages provide information about the biology and history of *C.elegans*, as well as up to date technical procedures and bioinformatical tools and information (Antoscheckin & Sternberg, 2007).

Plant DNA repair is less extensively studied, but they are also prone to alkylating agents and show many of the same DNA repair mechanisms as the nematodes and therefore underlines the relevance of *C.elegans* in ecotoxicological questions (Tuteja *et al*, 2001).

1.4.3.1. DNA damage and repair in *C.elegans*

The ability to overcome DNA damage and to have a strong repair system is crucial to any organism. The knowledge of DNA damage response in *C.elegans* somatic cells is still limited, but DNA damage response in the germ cells of has been extensively studied (O'Neill & Rose, 2006). This makes *C.elegans* a highly suited organism for studying compounds that might have an impact on genetic stability. The main types of DNA repair in *C.elegans*, as in all

other species, are direct reversal, nucleotide excision repair, base excision repair, mismatch repair, non-homologous end joining, homologous recombination repair and chromosomal structure surveillance (O'Neill & Rose, 2006).

DNA damage checkpoints in the germ line.

As described in section 1.4.2., there are two types of DNA damage response in the germ line; the mitotic germ nuclei proliferation arrest and the CEP-1-mediated apoptosis of damaged meiotic nuclei (O'Neill & Rose, 2006). When DNA damage occurs, germ cells in the mitotic region arrest their proliferation to allow time for DNA repair (O'Neill & Rose, 2006). DNA damaged germ cells in the meiotic region undergo apoptosis by the conserved germline apoptotic machinery (O'Neill & Rose, 2006). *C.elegans* CEP-1 (ortholog of the *p53* tumour suppressor gene in humans), is required for DNA damage-induced apoptosis (figure 11). In response to ionizing regulation, CEP-1 is regulated by the checkpoint genes *mrt-2*, *hus-1*, *rad-5* and *clk-2* (O'Neill & Rose, 2006). The CEP-1 protein up-regulates the " egg laying abnormal" EGL-1 protein and CED-13, which in turn opens up the membrane and causes release of apoptotic signalling components (Lant & Storey, 2010).

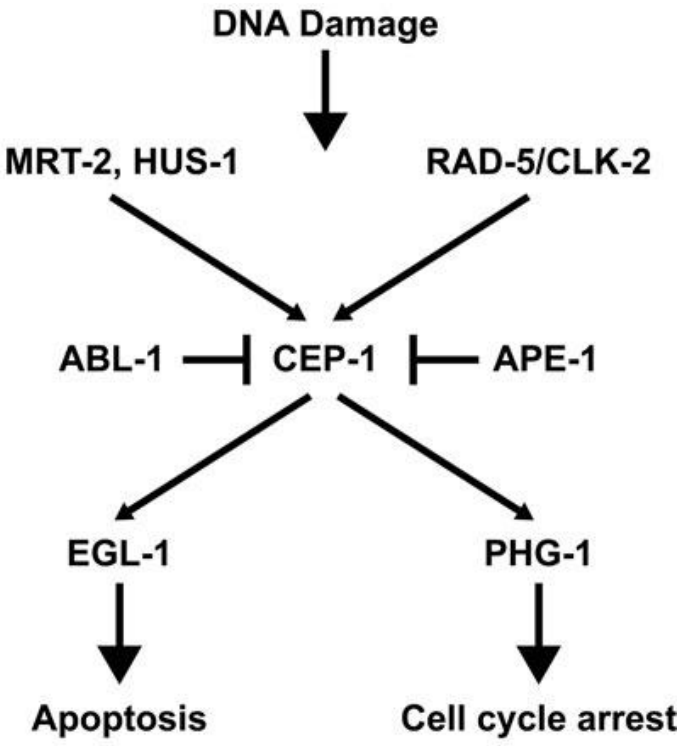


Figure 11. Overview over the checkpoint genes involved in DNA damage induced germ cell apoptosis. Adapted from O'Neill and Rose, 2006, www.wormbook.org

1.4.3.2. Stress response in *C.elegans*

One of the ways in which *C.elegans* can cope with stress (starvation, temperature, low level of oxygen) is by going into dauer stage as mentioned in section 1.4. In the dauer stage, larvae can survive up to ten times longer than adult worms (Lant & Storey, 2010).
The dauer stage is regulated by the insulin/IGF response pathway, which is initiated by the insulin and insulin-like growth factor (IGF). Insulin binds to the insulin-like growth factor and starts a signalling cascade propagated by PI3K. Activation of Akt (protein kinase B) by phosphorylation starts a number of downstream effects such as cell cycle control, glycogen synthesis and apoptotic suppression allowing a lowered metabolism (Lant & Storey, 2010).

C.elegans lifespan and development is regulated by the "Signal transducer and activators of transcription" (STAT) family (Lant & Storey, 2010). Mammalian STAT signalling controls a large number of developmental processes, and share many similarities with *C.elegans* development. They also play an important role in dauer regulation (Lant & Storey, 2010).

Under some stressful conditions, the cells can undergo a process called autophagy. Autophagy is an alternative to complete cell death, a type of cell shrinking whereby the cell organelles degrade and are recycled (Lant & Storey, 2010).

In addition to genotoxic and oxidative stress affecting the *C.elegans* genes, environmental stressors may also affect the *C.elegans*' behaviour. Environmental stressors may affect behaviour such as egg laying rate and brood size, which is a reflection of the nervous system (Hart, 2006).

2. Objectives

The aim of this thesis was to evaluate the toxicity of DMNA and NDMA, both the acute, chronic and molecular level of toxicity, and to compare the effect of the two substances to see if they have the same effect. Endpoints that were measured were brood size, reproduction, maturation, nematode size, fertility and DNA damage-induced apoptotic corpses. This was done by using standardised methods, some with modifications. It will also evaluate *C.elegans* as a research organism in modern ecotoxicological challenges.

3. Materials and Methods

3.1 Materials

3.1.1. Chemicals

Distilled water (dH_20)

Lysogeny broth (LB)-medium (for details see appendix)

Calcium chloride solution. 1 mol/l $CaCl_2$. 147 g $CaCl_2 \cdot 2H_2O$ in 1000 ml water

Cholesterol stock solution, 100 mg powdered cholesterol in 100 absolute ethanol (>99% purity)

Magnesium sulfate stock solution, 1 mol/l $MgSO_4$. 247 g of $MgSO_4 \cdot 7H_2O$ in 1000 water

Potassium hydroxide, KOH, pellets

Potassium phosphate buffer, 1 mol/l KH_2PO_4

Nematode growth medium (NGM) agar:

M9 medium (for details see appendix)

Bengal Rose stock solution, 300 mg/l

Benzylcetyldimethylammonium chloride monohydrate (BAC-C16) stock solution

Glycerol

Dimethylnitrosamine (NDMA, Cas number C15604000, lot 90225, Dr. Ehrenstorfer Gmbh)

Dimethylnitramine (DMNA, Cas number 98132-60 G, batch 11152, Chiron AS)- purity?.

3.1.2. Equipment

Incubator, $20\ ^0$ C, $31.5\ ^0$ C, $70\ ^0$ C

Shaker

Autoclave

Sterile Petri dishes (3 cm and 10 cm)

Drigalski spatula (aluminium wire)

Erlenmeyer flasks

Plastic vials

Freezer

Micropipette

Microscope, 100-fold magnification

Thermometer

Stereomicroscope

Clean bench

Balance, 0.001 g resolution

Multidishes, with 24 (3.5 cm^3) wells

Centrifuge

Piston pipettes, 10 ml to 100 ml, 100 ml to 1000 ml

PCR machine

EpiFluorescence scope

3.2. Methods:

3.2.1. Culturing of *Caenorhabditis elegans*

Strains of *C.elegans* were obtained from the *Caenorhabditis* Genetics Centre (CGC) in Columbia, MO (USA) and are free of charge for educational purposes and non-commercial research (Hope, 1999). The worms were frozen in liquid nitrogen by using glycerol-containing media, allowing long time storage of the worms (Hope, 1999).

The *C.elegans* was cultured on petri plates with NGM medium seeded with the *E.coli* strain OP50 as the food source. The *E.coli* OP50 is a uracil autotroph, a strain with reduced growth on NGM plates that is well suited for easy observation of the worms (Hope, 1999).

3.2.2. Preparation of NGM plates

The NGM medium (see materials) was melted in a microwave for 10 minutes until all agar had dissolved, followed by a water bath to cool the medium to 55 ^{0}C. Using sterile conditions, the following components were added under shaking in the following order (for 150 ml NGM): 150 µl $CaCl_2$, 150 µl Cholesterol solution (5 mg/ml in 98 % Ethanol), 150 µg 1M $MgSO_4$ and 3.75 ml K-Phosphate pH 6.0. The solution was then added to plates, making sure that the bottom of the plate was completely covered, and the plates with NGM medium were left to solidify for about 24 hours.

3.2.3. Transferring worms to a fresh plate

To prevent starvation, nematodes were transferred to new plates by a technique called *chunking.* A small piece of the NGM agar containing worms was cut out of the plate using a sterile scalpel, and carefully transferred to a plate with fresh bacteria.
When transferring a single worm at a time, worms were gently picked up with a Drigalski spatula (aluminium wire) using *E.coli* as an adhesive source for the nematodes.

3.2.4. Obtaining synchronous stage larvae

Synchronization of the population was achieved by alkaline hypochlorite treatment. In brief, the nematodes were collected by M9-buffer in a 15 ml Falcon tube, centrifuged at 1500 rpm for two minutes and the pellet was then diluted with 3.5 ml M9-buffer. A hypochlorite solution consisting of 384 µl NaClO, 500 µl 5M NaOH and 616 µl M9 was added, incubated for six minutes with vortexing every two minutes to dissolve the worms. At the end of the treatment, the eggs were pelleted by centrifugation at 1500 rpm, for one minute, the supernatant was removed and the pellet resuspended in 5 ml of M9-buffer. The washing of the pellet was repeated three times before the eggs were then resuspended in 1000 µl M9 buffer and transferred to a NGM plate, placed on a shaker and incubated at 20 ^{0}C. The following day synchronised L1 staged worms could be transferred to an OP50-seeded plate.

3.2.5. Generating males by heat shock

In order to increase the number of males in the F1 generation, small plates were set up with L4 worms. Approximately 10 L4 individual worms were transferred to a small plate, which was heated to 31 ^{0} C. After the heat shock treatment the worms were incubated at 20 ^{0} C leading to an increase in male frequency in the F1 generation.

3.2.6. Mounting animals for live imaging.

To observe alive worms in a Nomarski DIC microscope, a special slide was prepared. If the worms had been put simply between a cover slip and slide, they would have been crushed. Therefore, an agarose pad was prepared in the following way:
First, 4 % agar was made of agarose and distilled water. While keeping the agar warm and melted, one clean glass slide was put between two slides with labelling tape on them. The two slides with labelling tape gave a minor height difference compared to the clean slide. A drop of hot agar was pipetted on the clean slide, and another clean slide was placed across the bottom slide to create a flat agar pad. After approximately two minutes, the side slides and the top slide were carefully removed, creating an agar pad on with to put nematodes for microscopy. To ensure that the nematodes would lie still, they were drugged with 10 mM Levaminasol. Approximately 5 µl of Levaminasol was pipetted on the agar pad before the nematodes were pipetted on and carefully covered with a glass slide.
This technique was used when scoring apoptotic corpses in the germline of the nematodes.

3.2.7. Life stages characterisations

The *C.elegans* life stages characteristics were determined for 20 synchronized L1 staged nematodes in two small plates (3 cm^2). Approximately 20 L1 were transferred to each of these plates, and incubated at 20 ^{0} C. During the following days, the different stages of the worms were identified according to criteria proposed by Altun and Hall (2009).

4. Toxicity assays

4.1. Acute toxicity

4.1.1. Acute exposure assay (modified from Przybysz *et al*, 2007).

A population of wild type Bristol N2 worms were hypochlorite-treated according to standard procedures described in chapter 3.2.4. The worms were exposed to various concentrations of the test compounds NDMA and DMNA in a 24-well plate for 1h. The M9-buffer solution was

used as a negative control. After one hour the worms were transferred to OP50 seeded plates and allowed to recover for 24 hours. The following day, survival was scored. Worms that didn't respond to a touch-provoked stimulant with an aluminium wire were scored as dead. A concentration-response curve was made by non-linear regression to assess the survival rate.

4.2. Chronic toxicity

4.2.1. L4 assay – impact on fertility and development.

The L4 assay is an excellent way to score several phenotypes in one experiment and gives a good indication of toxicity. In this assay, L4 worms were exposed to various concentrations of DMNA and NDMA. The ability of the P1 generation worms to lay eggs was monitored, as well as the ability of the F1 generation to reach L4 stage and to reproduce (become gravid). NGM-medium with various concentrations (0 mM, 0.1 mM, 0.5 mM, 1 mM, 5 mM, 10 mM, 20 mM, 40 mM, 60 mM and 80 mM) of DMNA or NDMA were prepared in 24-wells and allowed to dry for approximately 1.5 hours. Each concentration of NGM-medium plus toxicant was first prepared in 50 ml Falcon tubes, then two wells were filled with 1500 µl of the medium for each concentration. When the medium had dried after approximately 3 hours, 15 µl OP50 *E.coli* was seeded on the wells and allowed to dry for approximately 2.5 hours. Two L4 staged worms were placed in each well. After 24 hours, the number of eggs was scored and the health of the adult worm was observed. The adult worm was removed, and the eggs were allowed to hatch. The eggs were followed after 48, 72 and 96 to see if they reached L4 stage and to observe the rate of fertility of the F1 generation.

4.2.2. ISO 10872:2010 "Water quality – Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans*"

In order to comply with international standards a test by the International Standard Organization (ISO) was included.

The chronic toxicity of *C.elegans* was determined by the ISO protocol ISO10872:2010: "Water quality – Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans*".

The principle of this assay was to expose juvenile nematodes to an environmental toxicant for a period of 96 hours and compare the outcome with a control. After this time the worms were killed by heat and the length, number of eggs and reproduction rate are measured. They were

dyed pink for a more easy recovery. Toxicity was calculated from the percentage decrease of these end points.

Approximately 30-40 L1 worms were transferred to each 24 well containing medium and concentrations 1 mM to 20 mM) of the test compounds to a total volume of 500 µl, one positive control and media as a control. The nematodes were incubated on an orbital shaker at 20 ^{0} C for 96 hours, added the dye Bengal Rose to facilitate easy scoring and recovery. The worms were then heated at 70 ^{0} C for 10 minutes to kill the worms in a way that facilitated elongation of the nematodes for the measurement of the length and number of the fertile (gravid/non-gravid) organisms under a stereomicroscope. The length was measured using AuxioVision software. The test was done three times.

4.2.3. Impact of DMNA and NDMA on brood size.

Heat shock is known to affect brood size in *C.elegans*. Measuring the change in heat stress resistance after exposure to environmental factors (such as chemicals and radiation) might give a good indication of toxicity. The goal of this assay was to find out if chronic exposure with DMNA and NDMA influenced the heat stress resistance and increased the male rate. This assay analysed the brood size in 3 cm plates with NGM agar. One control, three plates with 0.5 mM, 5 mM and 20 mM DMNA. The same concentrations were used for with NDMA. They were seeded with OP50 the morning after and allowed to dry for 3 hours. N2 Bristol worms were synchronised by bleaching according to standard procedure the day before, so that L1 staged worms could be used for the experiment. Approximately 20-30 L1 worms were transferred to each of the small plate. On day 3, when the L4 stage was reached, 10 worms were transferred to a new plate and heat shocked at 31 ^{0} C for 5 hours. One control group was also heat shocked, and the other was not heat shocked. In addition, one plate with DMNA and one plate with NDMA were not heat shocked, these control plates were kept at 20 ^{0} C. After the heat shock was completed, the worms were again incubated at 20 ^{0} C. The following day, one adult worm was transferred to a new OP50 seeded plate in three duplicates. The number of eggs were monitored and scored, and the worm was transferred to a new plate each day. The F1 generation was monitored to check for an increased male rate.

4.2.4. Impact of DMNA and NDMA on heat shock resistance.

When subjected to toxic chemicals, stress response genes are expected to be upregulated which might make them more resistant to heat stress (ref). To observe if our test chemicals

cause increased tolerance to heat, N2 Bristol strain worms were grown on NGM-plates seeded with OP50, and bleached according to standard procedures when there were enough adults and eggs on the plate. The following day, L1 worms were transferred to plates containing DMNA or NDMA with the concentrations 0.5 mM, 5 mM and 20 mM.
After three days, when L4 stage was reached, ten L4 worms were transferred to three different plates. The plates were incubated at 35 ^{0} C and monitored every hour. Worms were scored as dead when they did not respond to a touch provoked stimulus. This was done for DMNA, NDMA and two controls, one untreated and without heat shock, the other untreated with heat shock. The experiment was done twice.

4.2.5. Accumulation of spontaneous mutation

Alkylating agents are expected to be mutagenic. It was therefore of interest to observe the rate of spontaneous mutations according to procedures described by Vassilieva and Lynch (1998). In essence, one plate with wild type Brystol N2 strain was cultured on a OP50-seeded NGM plate and kept at 20 ^{0} C for 3 days until L4 stage was reached. At the beginning of the experiment, ten randomly chosen L4 worm from the G_0 generation were placed on a 3 cm NGM plate with DMNA, this was repeated in triplicates. As a control, ten randomly chosen worms in the G_0 generation were placed in ten PCR tubes containing a lysis buffer, consisting of 50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl, 0.45% NP40 and 0.45% Tween20, which were lysed in the PCR machine. The lysates were kept at -80^{0} C until the end of the experiment.

The NGM-plates were prepared according to standard procedures described in section 3.2.2, except in this assay NGM-plates were prepared with 0.5 mM DMNA or NDMA, in addition to one series with no drug for control. Every fourth day, one L4 worm was placed in a fresh NGM-plate with drug and allowed to hatch, the same was done with the control. The adult worm was removed the next day, and three healthy L4 worms were chosen from the next generation on day 4.

When the 10th generation was reached, 15 worms from each plate were lysed as mentioned earlier. Twenty lysates of worms exposed to DMNA, NDMA and twenty untreated lysates were chosen randomly for isolation of DNA and PCR amplification. Primers used for the

amplification were based on previous experiments from Vassilieva and Lynch (1998), known for their accumulation of mutations.

Primers used were:

ZK337_F: CGACATTTTGGGAAGTGTTTCG	Melting temp 53 ^{0} C
ZK337_R: AGCAGAAGAACGGAGCATCGTC	Melting temp 57 ^{0} C
M106_F: TGAAAAGTTCGCCACAAGTCG	Melting temp 52 ^{0} C
M106_R: TCAGTCAGTCAGTCACTCCAATCG	Melting temp 57 ^{0} C
F59F5_F: GACTTGCTCTTGGTGAACTTCTCG	Melting temp 57 ^{0} C
F59F5_R: GCGGGATAATGAATCTCTACGGC	Melting temp 57 ^{0} C

The melting temperatures were looked up by the oligocalculator (http://www.basic.northwestern.edu/biotools/OligoCalc.html). Ideally, the melting temperature for the forward and reverse primers would be the same, but since they were not a gradient PCR was run to find the optimal melting temperatures.
Primer pair one (ZK337_F and ZK337_R) was run at 58.9 ^{0} C, primer pair two (M106_F and M106_R) at 60.9 ^{0} C and primer pair three (F59F5_F and F59F5_R) at 54.3 ^{0} C.
The PCR reaction was set up by a kit of 22.5 µl AccuPrime Mix, 0.5 µl 10 µM Forward Primer, 10 µM Reverse Primer and 1.5 µl of the lysate (template). After the PCR had run

The PCR products were sent to the GATC Biotech Centre in Germany for sequencing. The GATC had to do a clean-up (buffer exchange and primer removal). After a few weeks, the sequences were ready to be downloaded from their webpage, analysed in free-ware, and the control and treated samples were aligned in Clustal W and compared.

4.2.6. Inducing apoptosis in germ cells

Apoptotic cells in the germ line can be visualized by a method using *C.elegans* worms containing the protein CED-1::GFP (Gartner *et al*, 2008). This protein shows a characteristic structure of somatic cells surrounding the apoptotic cells. Gartner *et al* showed that alkylating agents can induce genotoxic stress and apoptosis in germ cells. They found that the alkylating agent ENU (N-nitroso-N-ethylurea) increased the number of apoptotic cells in the same way radiation did. Germ cell apoptosis occurs mostly in the loop (parchytene zone), and is known as a good indicator of genotoxic stress.The increase of apoptotic germ line cells is a well-developed method to study the toxicity of alkylating agents. The use of CED-1::GFP

expressing transgenic animals gives a powerful indication of the test chemicals' ability to induce apoptosis. CED-1::GFP is a marker strain that visualises the dying cells better as they are engulfed by the sheath cells surrounding the germ line.
The CED-1::GFP was grown on NGM-plates and synchronized by bleaching according to standard procedures. The test was performed by exposing the worms for concentrations (40mM, 60 mM, 80 mM and 100 mM) of DMNA or NDMA in a liquid medium (M9-buffer with OP50) for 24 hours, incubated on a shaker. M9-buffer with OP50 was used as a negative After 24 hours, the worms were put on agarose pad (see section 3.2.6.), and scored for apoptotic corpses under a Differential Interference Contrast (DIC) microscope using fluorescence light and a 64x magnification.

5. Statistical and graphical methods.

The data was collected and stored in Excel. The average, standard deviation, SEM (standard error of the mean) and deviation from control were calculated here (raw data in the appendix). Data was also plotted into GraphPad 4, and all the graphs were drawn in this program. The EC50 at a 95% confidence interval was calculated in GraphPad 4.

6. Results

The aim of this thesis was to evaluate the toxicity of DMNA and NDMA, both the acute, chronic and molecular level of toxicity, and to compare the two substances to see if they had the same effect. This was done by both using standardised methods, some with modifications.

6.1. Life stages characterizations.

As the toxicity of compounds can vary greatly with the developmental stage of the test-organism, it was important to first get familiarized with the life cycle of the organism and to recognize different developmental stages. Figure 10 shows the stages of the nematodes day by day:
After overnight incubation (day 0) all the eggs had hatched, and all the worms were L1 stage (approximately 250 µm in size). The stages L2 and L3 were very difficult to distinguish by size and were scored as one. On the third day L4 stage was easily distinguished by the white mark at the vulva region. Thus, figure 12 illustrates the invariant development of early *C.elegans* embryos where development from L1 to L4 could be monitored.

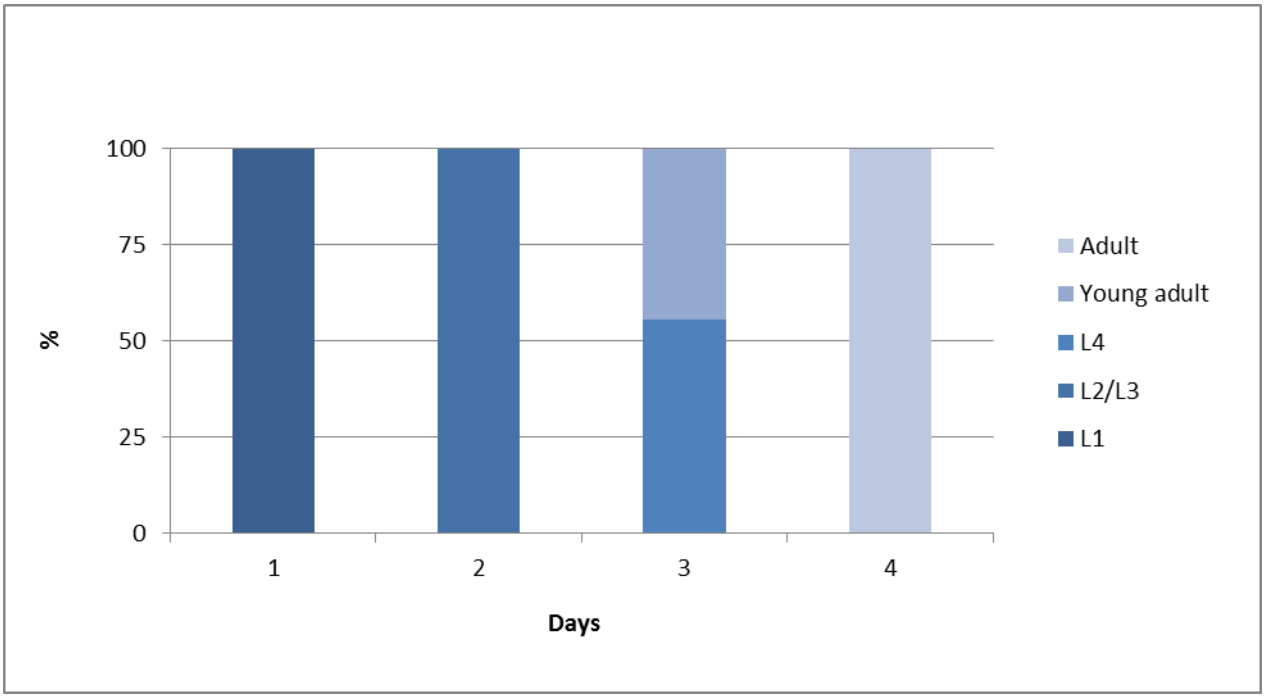


Figure 12. This histogram demonstrates the synchronous development of the C.elegans. With the exception of the third day, when certain individuals grow into young adults faster than others, showing that their development is very predictable and invariant.

6.2. Acute toxicity

Exposing the nematodes for one hour showed no acute toxic effects for the concentrations used in NDMA (figure 13), and low acute toxicity after exposure to DMNA (Figure 13). The nematodes exposed to NDMA were scored as alive as they responded to touch provokement. However, after exposure to DMNA some adverse effects were seen. The toxicological endpoint in this assay (death) was seen first at concentrations above 333 mM

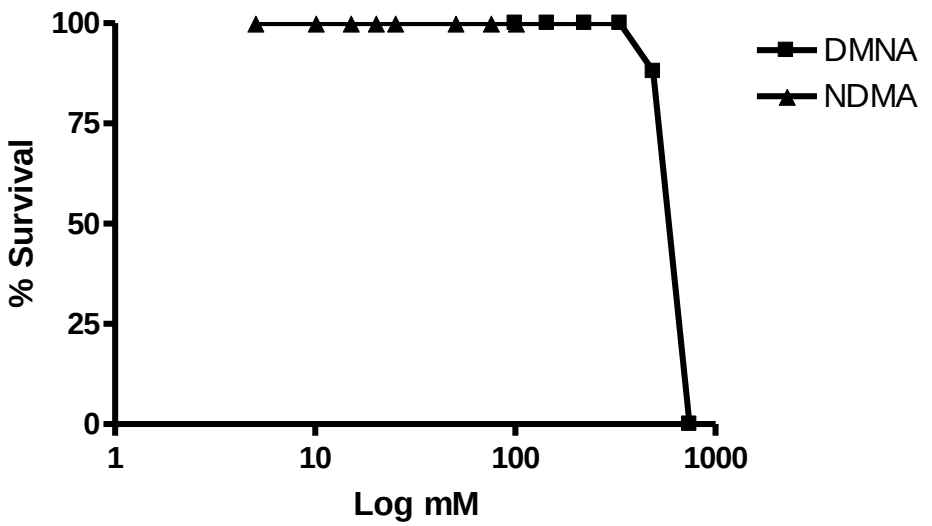


Figure 13. Acute toxicity after one hour of exposure of DMNA and NDMA. Approximately 30-40 adult worms were exposed to increasing concentrations of DMNA (A) or NDMA (B) for one hour in liquid medium and then transferred to seeded plates for 24 hours to recover. The fraction of living worms were measured after 24 hours and plotted as % survival.

In conclusion, DMNA and NDMA had no acute effects on adult worms at physiologically relevant concentrations when exposed for one hour in a liquid medium

6.3. DMNA and NDMA impair developmental rates and fertility.

This assay evaluated three different phenotypes; the egg hatching rate, the development into L4 stage and the fertility. Figure 14 shows the impact of DMNA and NDMA on the egg hatching rate. For DMNA, the egg hatching rate was stable at approximately 100 % for two of the replicates at 0.1 – 20 mM, whereas the first replicate shows a lower egg hatching rate. The graph shows a negative trend, and dropped dramatically between 20 mM and 40 mM DMNA, from 74 % to 6.7 %. In NDMA, there was no such dramatic drop. There was only a minor decrease in the egg hatching rate, with the highest dose (80 mM) only 34.2 % less than the control (in contrast to DMNA, which had a 100 % decrease at 80 mM). The log EC50 for the egg hatching rate for DMNA was 10.9 and 40.5 for NDMA. The LOEC for DMNA was 0.5 mM and NOEC 0.1 mM, and for NDMA the egg hatching rate LOEC was 1 mM and NOEC 0.5 mM.

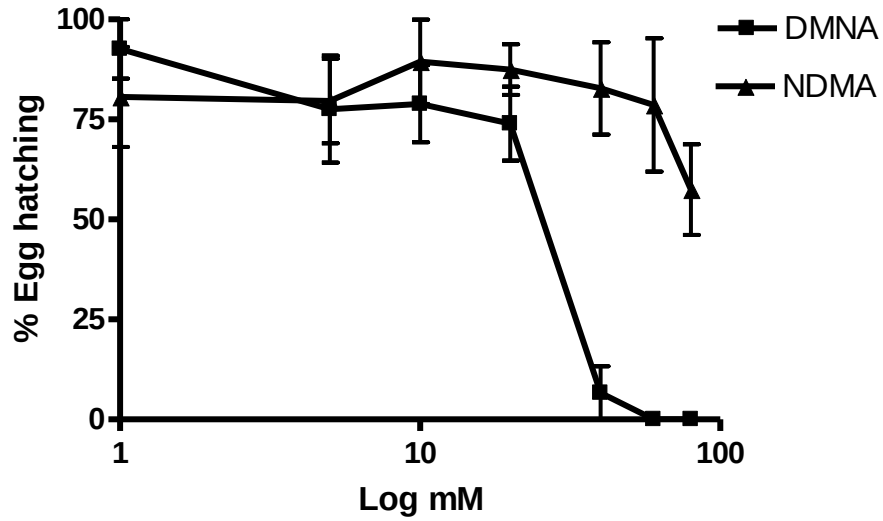


Figure 14. The effect of DMNA and NDMA on the egg hatching rate. At increasing doses of the test compounds, the egg hatching rate goes down dramatically. The effect is highest for DMNA than for NDMA.

Figure 15 below shows the effect of DMNA and NDMA on the ability of the F1 generation to reach L4 stage. This rate stays at 100 % until 20 mM, the Log EC50 for DMNA is 20 mM. For the NDMA sample the log EC50 is 40 mM. The NOEC for DMNA is 10 mM and LOEC 20 mM, for NDMA the NOEC is 20 mM and LOEC between 20 and 40 mM.

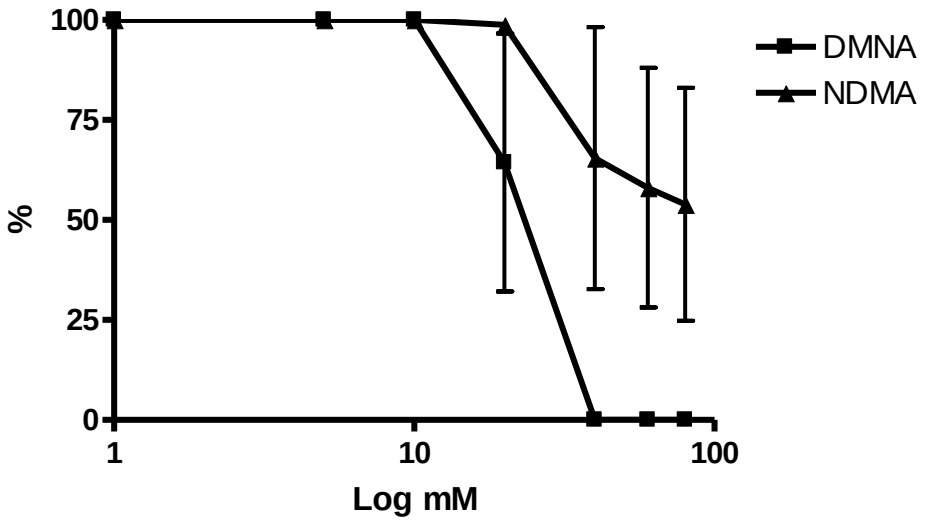


Figure 15. The effect of DMNA and NDMA on development of L4 stage. Again, the effect of DMNA is higher than the effect of NDMA.

The ability of the F1 generation nematodes to become gravid also decreases by increasing dose of test chemicals, this is also seen more clearly in DMNA (figure 16). At 20 mM DMNA, none of the nematodes are able to become gravid. For NDMA the toxicity is less (figure 15), but still leading to a 44.8 % decrease of gravid nematodes. The log EC50 for DMNA is 8.3, and log EC50 for NDMA 20. The NOEC for DMNA is 0.1 mM, and the LOEC is 0.5 mM. For NDMA, the NOEC is 0.5 mM and LOEC is 1 mM.

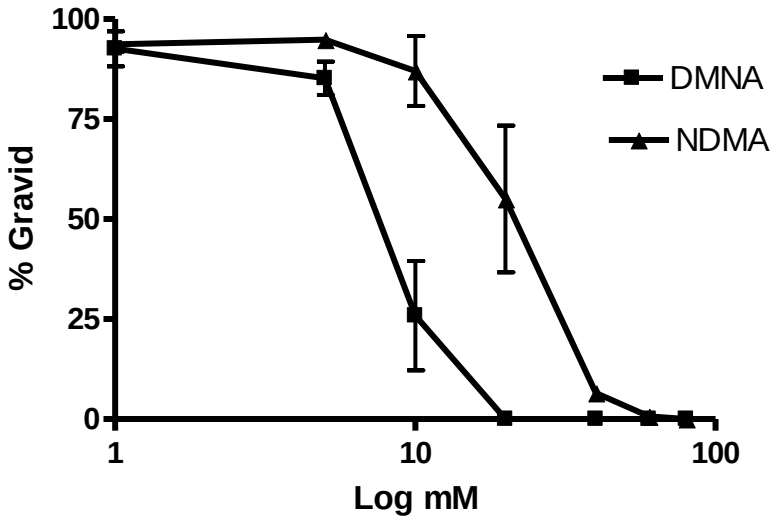


Figure 16. Comparison of the effect of DMNA and NDMA on the egg hatching rate. The graph shows a significant dose-response curve, with DMNA being more toxic than NDMA.

In conclusion; DMNA has a significant negative impact on the egg hatching rate, development into L4 stage and reproduction. NDMA also has a significant negative impact on egg hatching rate, development into L4 stage and reproduction, but the toxic effect is lower than for DMNA.

6.4. DMNA and NDMA impair growth and fertility in a liquid medium (ISO 10872)

As the use of standardised assay protocols are of great importance in toxicology, we included the ISO:10872 in our analyses. For DMNA there was a steady decrease in size correlated to the dose of drug (figure 16), with an average decrease of 39 % at the highest concentration (20 mM) compared to the control.

In NDMA the decrease in size was very limited up until the highest concentration (20 mM), which showed a decrease of 17.1 % compared to the control (figure 17). The log EC50 for effect on size in DMNA is 5.2 and the log EC50 for NDMA 15.99. The NOEC is 1 mM and LOEC 5 mM for both DMNA and NDMA.

The fertility decreases in both DMNA and NDMA, but DMNA shows the largest effect.

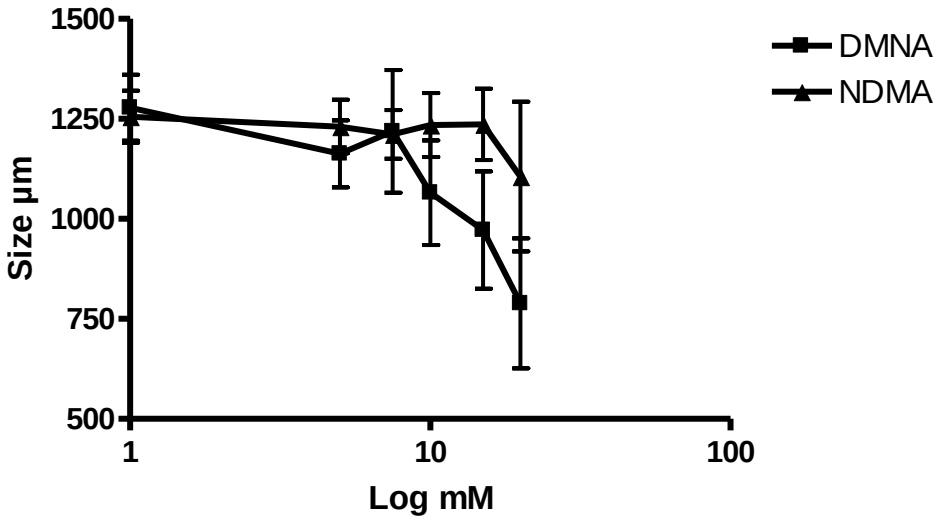


Figure 17. The reduction in nematode length after having been exposed to DMNA or NDMA for 96 hours. Exposure to DMNA (square points) shows a significant decrease in nematode length, whereas the decrease in nematodes' length exposed to NDMA(triangle points) is minor.

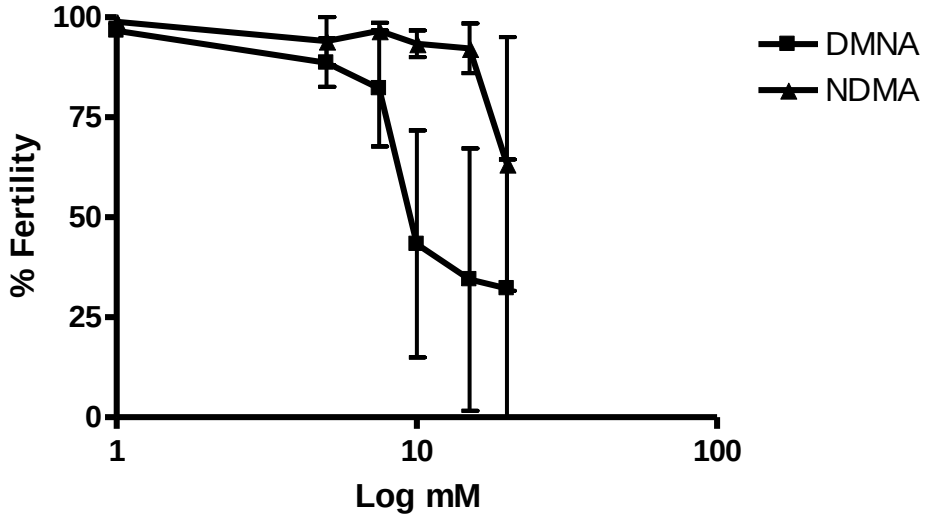


Figure 18. Comparison of the effect on fertility in the nematodes exposed to DMNA and NDMA for 96 hours in a liquid medium (ISO 10872). When comparing the two chemicals, DMNA (square points) lead to a more negative impact on fertility than NDMA (triangle points).

In conclusion; DMNA and NDMA both have a negative impact on growth and fertility, but the strongest negative effect is seen in DMNA.

6.5. Multiple stressors: measurement of brood size by heat shock after treatment with DMNA and NDMA.

As both ISO and L4 assay indicated a negative impact on fertility, we decided to quantify the negative effects on brood size. The brood size after exposure to DMNA from L1 to L4 stage was scored and plotted into a diagram (figure 19a). The same was done for NDMA (figure 19b). The final three columns (DMNA or NDMA "- HS") showed the effect of just DMNA or NDMA exposure, but without heat shock. Worms exposed to 0.5 mM DMNA plus heat shock (+HS) showed a marked decrease in brood size, the same was seen for 0.5 mM NDMA plus heat shock. At 5 mM NDMA (+HS) the worms showed a less negative effect on their brood size (larger brood size), indicating increased heat resistance. DMNA showed the same negative impact on brood size as in 0.5 mM, but there was no significant difference at 5 mM. At 20 mM, the brood size dropped dramatically for both chemicals (+HS).

a)

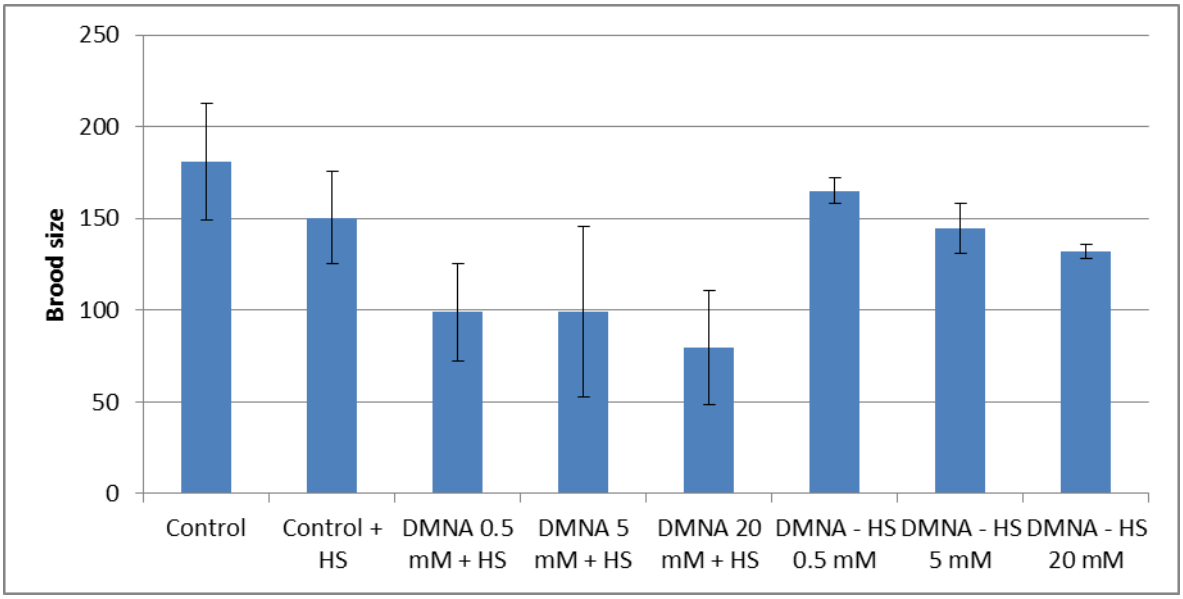


Figure 19 a) Nematodes grown on plate with DMNA since L1 stage show a reduction in brood size with heat shock (+HS)and without(-HS) heat shock.

b)

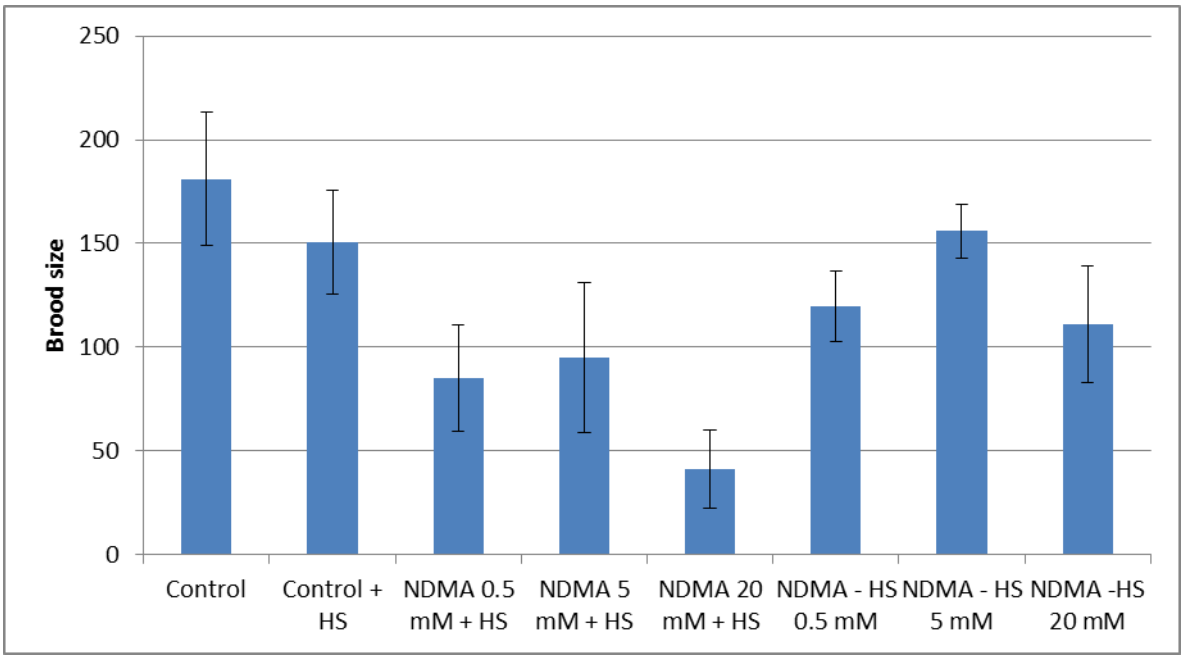


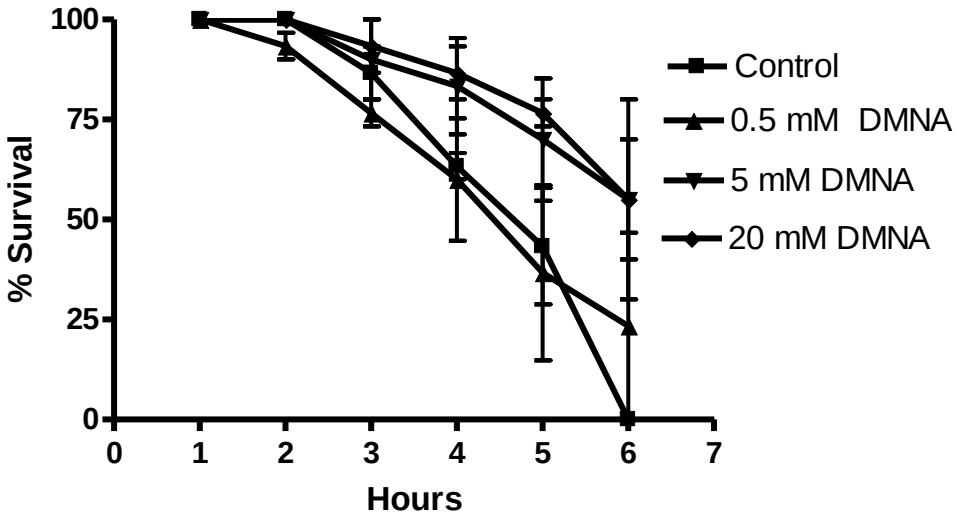
Figure19 b) Nematodes grown on plates with NDMA since L1 stage also show a reduction in brood size.

The conclusion is that the brood size is negatively influenced by long term exposure of DMNA or NDMA, and that stress response is activated and but the brood size is significantly reduced.

6.6. Multiple stressors: activation of stress genes by treatment with DMNA and NDMA followed by heat shock.

This was also a multiple stressor assay, this assay scored for heat shock survival after a L1 – L4 stage exposure to DMNA or NDMA. The survival curves show that in both DMNA (figure 20a) and NDMA (figure 20b) the two higher concentrations (5 mM and 20 mM), show a higher resistance to heat stress than the lowest concentration (0.5 mM) and the control.

a)



b)

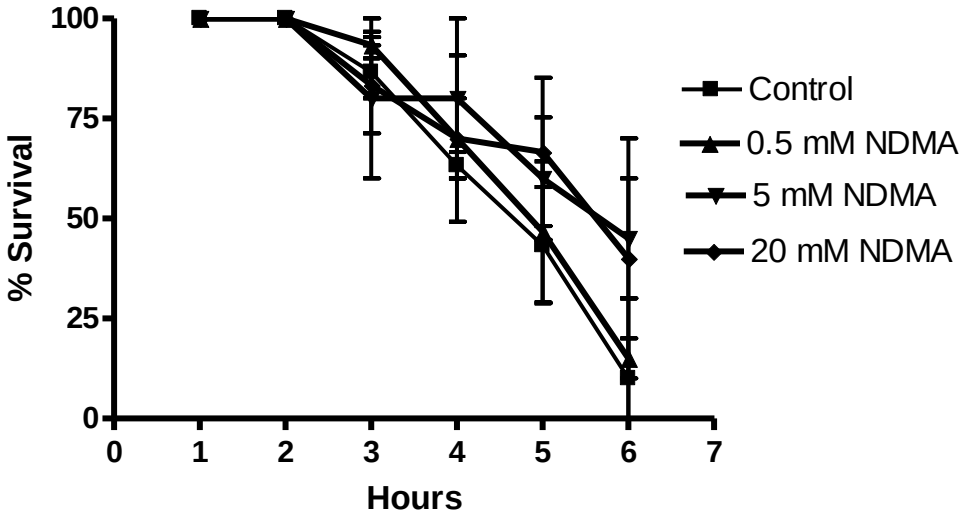


Figure 20 a) DMNA: worms exposed to 0.5 mM behaved almost the same as the control. Worms exposed to 5 mM and 20 mM showed increased tolerances to heat stress.
b) NDMA: worms exposed to 0.5 mM behaved almost the same as the control. Worms exposed to 5 mM and 20 mM showed increased tolerance to heat stress (but with a less significant difference then DMNA).

The conclusion is that chronic exposure to DMNA or NDMA increases the heat shock tolerance because in the P0 generation stress response genes are activated.

6.7. Genetic toxicology: Spontaneous mutations

Based on a spontaneous mutation accumulation done by Lynch & Vassilieva (1998), we decided to quantify spot mutations in three regions. Quantification of the number of spontaneous mutations was expected to give a good indication of the compounds mutagenicity. Samples with primer number 2 (M106_F and M106R) were concidered as the best suitable for DNA sequencing, these PCR products were sent to the GATC centre for DNA sequencing. Unfortunately, the quality of the PCR products was too poor to be analysed, and no conclusions could be drawn from this experiment. The data is concluded in the appendix.

6.8. Genetic toxicology: induction of germline apoptosis.

A common endpoint for genetic toxicology is to measure increased apoptotic corpses in the germline. Figure 21 shows the number of apoptotic corpses after exposure to DMNA and NDMA indicating a correlation between DMNA or NDMA doses and level of DNA damage. The dose 60 mM DMNA gave a deviation of 8.2 apoptotic corpses on average compared to the control, in other words a 164 % increase. At 100 mM the number of apoptotic corpses was lower than the 60 mM dose.

The doses of NDMA showed a significant increase in apoptotic corpses, although less prominent than with DMNA. The concentration of 60 m.M. NDMA showed an average increase of 4.3 apoptotic corpses, or an 86 % increase compared to the control. The concentration of 100 m.M. NDMA gave an average increase of 5.6 (11.2 % increase), but the dose is still not toxic enough to cause cell cycle arrest as with the same dose of DMNA. Illustrations of the increase of apoptotic corpses are shown in figure 22 (c, g, and e) for DMNA and figure 22 (d, f and h) for NDMA. Figure 21a shows the negative control, and figure 22b) the positive control (ENU).

In conclusion, we showed that DMNA and NDMA are agents capable of causing apoptosis in the *C.elegans* germline.

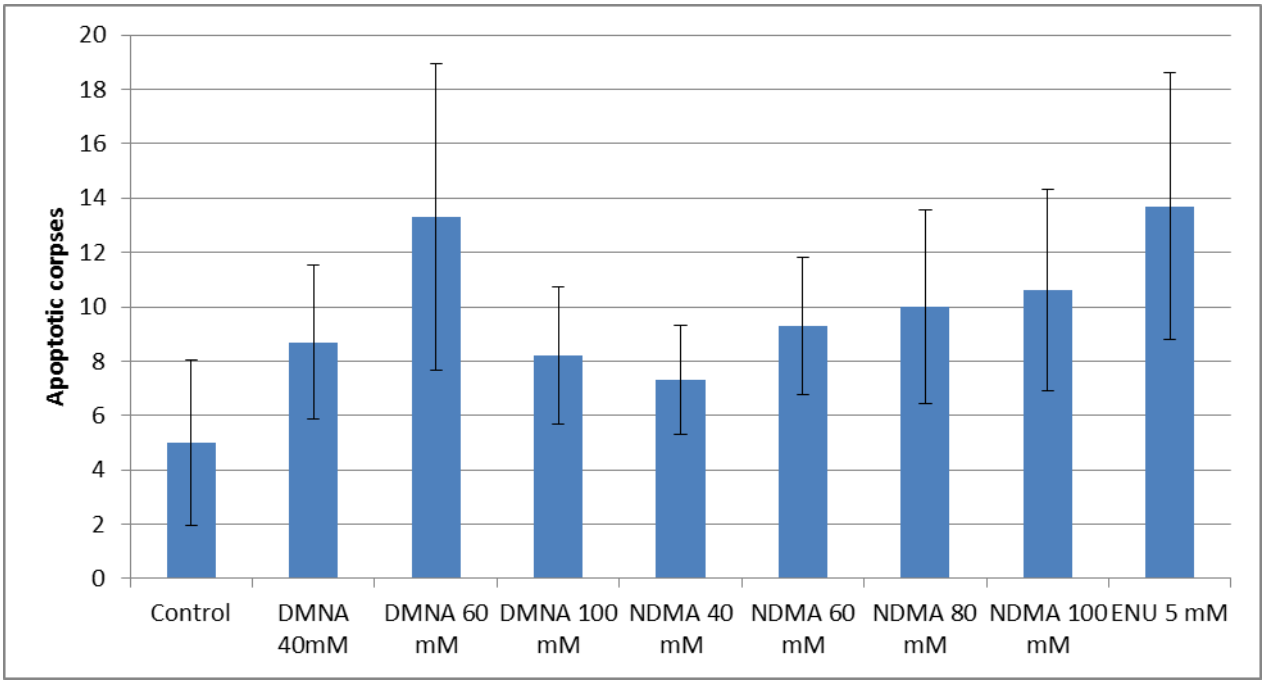
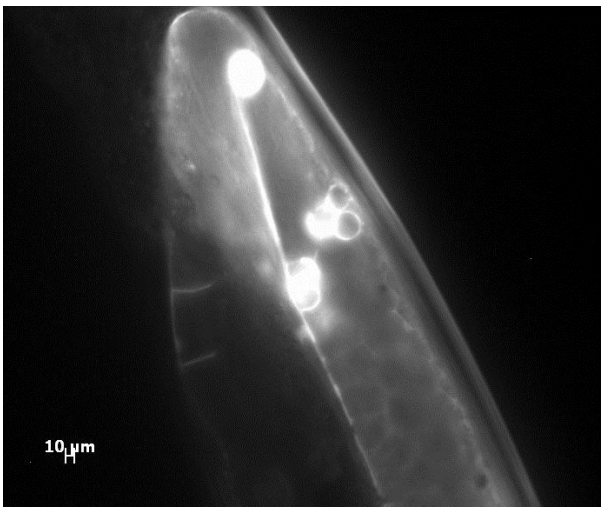
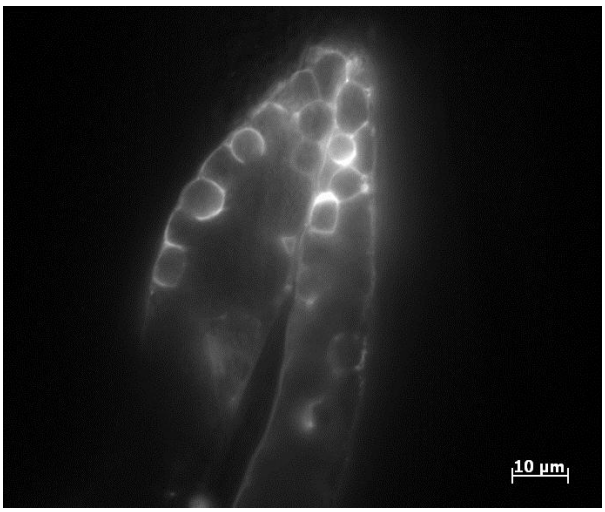


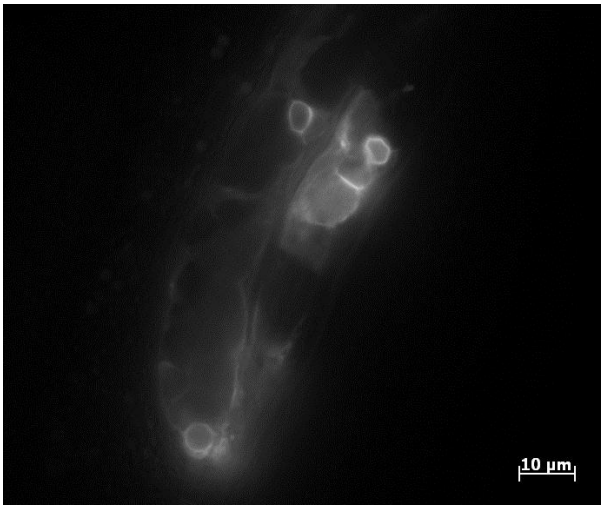
Figure 21. Shows the number of apoptotic corpses in worm gonads exposed to DMNA, NDMA and ENU compared to the control. The sample DMNA 80 mM was destroyed by handling, and had to be excluded from the results.



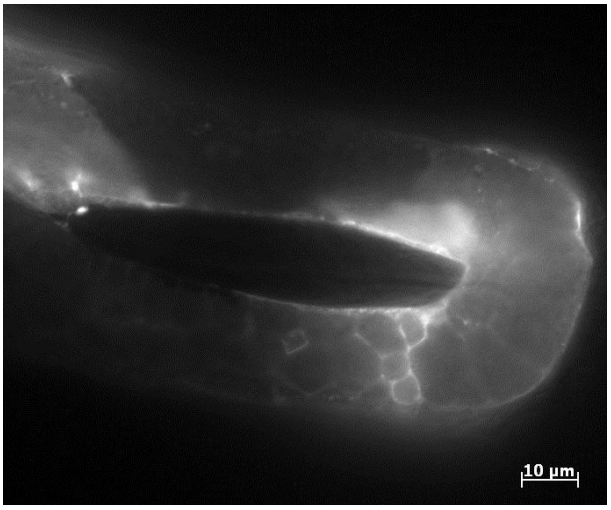
a) Control



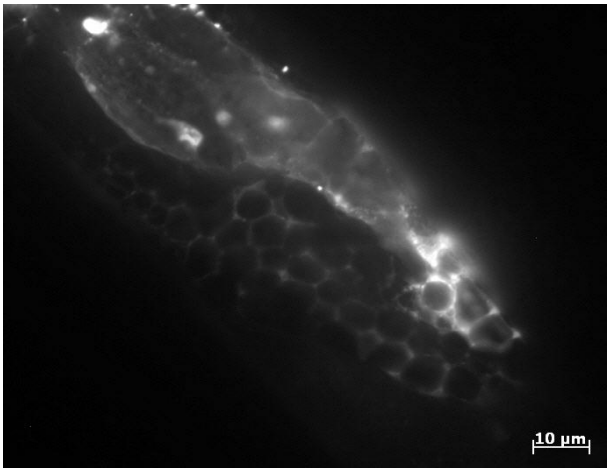
b) ENU 5 m.M.



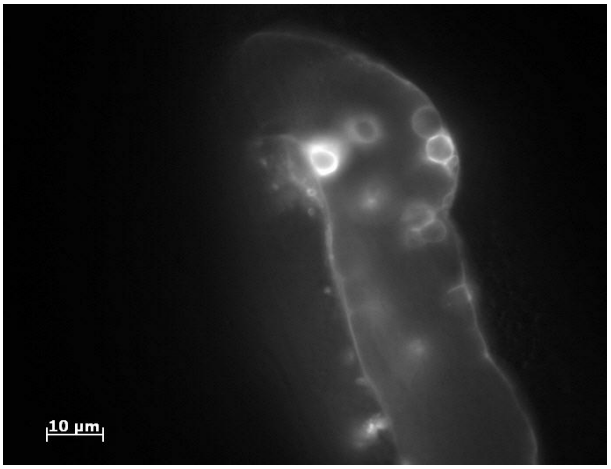
c) DMNA 40 m.M.



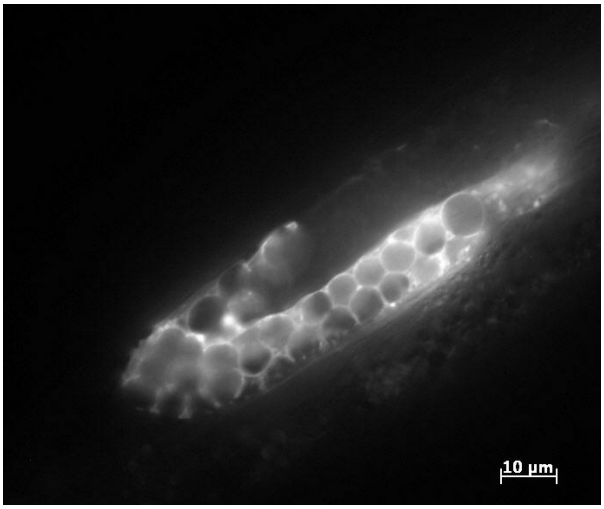
d) NDMA 40 m.M.



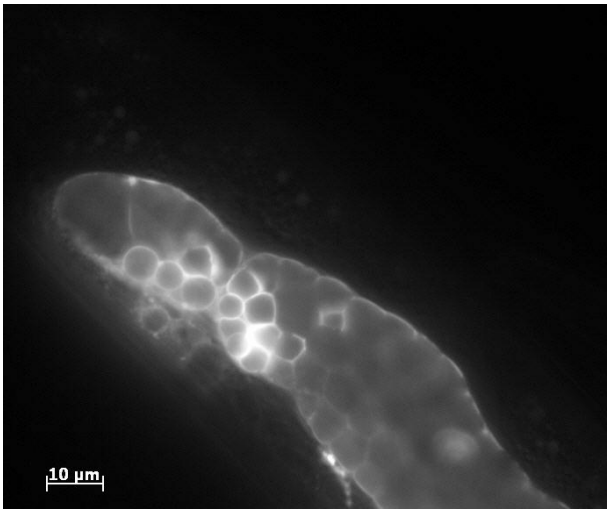
e) DMNA 60 m.M.



f) NDMA 60 m.M.



g) DMNA 100 m.M.



h) NDMA 100 m.M.

Figure 22. Illustration of the increase in apoptotic corpses in the death region in nematodes exposed to DMNA, NDMA and ENU, with a marked increase in apoptotic corpses at increased doses of drug. Pictures taken with a DIC microscope with fluorescent light, 63 x magnification, courtesy of Anneke Maria Bruno.

7. Discussion

The biological processes occurring in the different developmental stages illustrated above vary. For example somatic cell division and differentiation during embryonic development and germline development in the L3 to L4 transition are driven by different mechanisms, and the synchrony is looser in transition from L4 to adult. Toxicity of a drug might therefore be manifesting itself differently depending on the developmental stage of exposure. This biological complexity makes it likely that drugs might affect animals differently in different exposure regimes, making it necessary to evaluate different toxicity assays regimes. The histogram confirmed that the nematodes developed synchronously, and was easy to monitor.

The DMNA and NDMA showed little or no acute effect after one hour of exposure, and no acute toxicity could be measured in NDMA at concentrations of 5mM to 100 mM. Previously, The compounds have only been tested for acute toxicity in rats (Carcinogenic Potency Project database), which showed toxicity only at very high levels. While the results suggest that DMNA and NDMA are not acutely toxic in invertebrates, one hour of exposure will probably not be enough to induce this type of damage. DMNA shows signs of toxicity only at very high doses, and the acute toxicity of NDMA was not measurable at these concentrations.

In the combined chronic test; the egg hatching rate, maturation rate and fertility rate were selected as phenotypic parameters. These might be behavioural stress responses as described by Hart (2006). The L4 worms were exposed to DMNA or NDMA for 24 hours before they laid eggs. The results of this experiments vary a lot (large standard deviations), meaning the assay is difficult to reproduce. There can be numerous reasons for this; first, the test chemicals were blended into the agar (which solidifies quite fast), so there is a possibility that the distribution of the drug was uneven. Second, the assay was done two days in a row, using the same nematodes for these tests. The idea was to use the same generation of worms, but incubation overnight on a shaker might have led to hypoxia in the nematodes, affecting their resistance to the test chemicals.
The toxicity of NDMA varied considerably in several of the experiments, which might be because of its unstable nature. As mentioned in chapter 1.2., the NDMA degrades rapidly in sunlight. This was taken into consideration when handling the compound by storing it under dark conditions and handling it in dark surroundings (turning off the light) whenever practically possible. Nonetheless, there was a significant negative impact on these

phenotypes. If it can be assumed that the NDMA was not degraded by light, the DMNA is more toxic then the NDMA.

The ISO standard test measured the effect on size and fertility and showed a significant decrease in size and fertility for DMNA but not as large a decrease in NDMA. This is a broad assay suitable for quick screening of chemicals, but it has its limits. One of the problems with this assay is the food supply when keeping the worms incubated for 96 hours; some monitoring and supply of nutrients is required to prevent stress due to starvation. This might have led to change in the concentrations of the test substance. Another problem is keeping the worms on a shaker for 96 hours, this leads to stress response that might interfere with the results.

In nature, stressors rarely occur alone. Organisms are most often exposed to multiple stressors at the same time, which might be either additive (1+1 =2), antagonistic (1+1=0) or synergic (1+1 = 3). It was therefore of great importance to include at least one assay that addresses more than one environmental stressor at a time. It was also expected that treatment with alkylating agents would trigger stress response genes. The measurement of heat shock induced brood size showed that the lowest concentrations showed a heat stress effect, while there was an increased tolerance level at 5 m.M. for both DMNA and NDMA. The reduction of brood size NDMA than for DMNA in this experiment. This interesting observation indicates that 5 m.M. (0.45 mg/ml) activates stress response genes in *C.elegans*. The highest dose 20 m.M. (1.8 mg/ml) led to a decrease in brood size indicating cytotoxicity. The interesting part is that in both DMNA and NDMA there is an increase in brood size at 5 mM compared to 0.5 mM, indicating that these two chemicals both trigger activation of stress response genes.

The nematodes exposed to the higher doses of DMNA and NDMA showed a higher resistance to heat stress and were able to survive longer, indicating that stress response genes have been activated. In this experiment DMNA seems to activate more stress response genes then NDMA contrary to the previous assay, but this assay has a completely different end point (death).

Unfortunately, the PCR products sent to Germany for DNA sequencing were of poor quality and no results could be read from them. The most probable reason is that we did not spend

enough time optimising the PCR conditions. If we had run the gels for a longer period of time, it might have revealed that more optimisation was needed.

Previous studies with the alkylating agent ENU (N-ethyl-N-nitrosurea) has shown that alkylation leads to DNA damage and apoptosis, which can be monitored in germ line apoptosis assays (Stergiou and Hengartner, 2004). This was confirmed with DMNA and NDMA as they both significantly increased the number of apoptotic corpses in the gonads. Again, the effect appeared to be strongerfor the samples exposed to DMNA than the samples exposed to NDMA. The number of apoptotic corpses increased up until 60 mM. Interestingly, at 100 mM DMNA, the number of apoptotic corpses dropped to a lower count. This is probably due to the cells being arrested in the mitotic region, indicating that they are cytotoxic as well as mutagenic, meaning the cells never develop to become competent to undergo apoptosis. ENU has the highest number of apoptotic corpses (on average 8.8 corpses more than the control). This is to be expected as ENU is a directly activating carcinogen and known as one of the strongest inducers of alkylating DNA damage. Exposure to 60 mM DMNA increased the number of apoptotic corpses to 8.2 apoptotic corpses on average compared to the control at an average of 5 corpses per gonad arm, in other words a 164 % increase. At 100 mM the number of apoptotic corpses was lower than the 60 mM dose. In conclusion, we showed that DMNA and NDMA are alkylating agents capable of inducing apoptosis, suggesting they have genotoxic effects.

Since there is limited literature on the toxicity of nitramines, it has previously been assumed that NDMA is more toxic than DMNA. These experiments have shown otherwise, suggesting that DMNA has a larger ability to negatively influence growth, egg laying rate and fertility. At the same time, NDMA is an unstable chemical in air as mentioned, therefore better handling ways have to be considered when performing further testing to confirm this difference.

A more thorough experimental approach to study the impacts of nitrosamines and nitramines to terrestrial species is required. NILU'S report concluded that more research needs to be done on the effects of nitrosamines and other compounds created by the amines released by CO_2-capture technology.

To summarize, these experiments have indicated that DMNA may have a higher level of toxicity than NDMA, contrary of what has been assumed in previous studies. The assay counting apoptotic cells seems to be the strongest assay. It is also the assay that addresses the problem of environmental carcinogens directly.

With all the technology available and the great network online, it is absolutely advisable and of great importance to take this project to the next, molecular level. There is enough evidence to support that DMNA and NDMA are toxic on the molecular level which needs further attention.

9. Conclusion

This project has confirmed that DMNA and NDMA are genotoxic stressors that induce apoptosis, and these chemicals are also capable of influencing the organisms' growth and reproduction rate. The chemicals were not found to be very acutely toxic, however. The next steps in this line of research should consider to perform global gene expression analysis to uncover the up-regulation of stress response genes, for which *C.elegans* will be an ideal model organism.

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X. Appendix

X.1.Calculations:

Stock solution DMNA:

Molecular weight: 90.08 g/mol

50 mg/ml → 0.05 g/ml / 90.08 = 0.000555 mol.

c = 0.000555 mol/ 0.001 L = 0.555 M = 555 mM.

Stock solution NDMA

Molecular weight: 74.0 g/mol

50 mg/ml → 0.05 g/ml / 74.0 = 0.000675 mol.

c = 0.000675 mol/0.001 L = 0.675 M = 675 mM.

Acute toxicity:

Based on these calculations, a 24-well with the following concentrations was set up:

For DMNA:

Concentrations mg/ml	**0**	**9**	**13**	**20**	**30**	**44**	**67**	**95**
Concentration (mM)	**0**	**99,9**	**144**	**222**	**333**	**488**	**744**	**1050**
Dimethylnitramine(µl)	0	45	65	100	150	220	335	475
OP50 + L4 worms + M9 mix (µl)	500	455	435	400	350	280	165	25

For NDMA:

Concentration (mM)	**0**	**5**	**10**	**15**	**20**	**25**	**50**	**75**	**100**
Dimethylnitros-Amine (µl)	0	3,7	7,4	11,1	14,8	18,5	37,0	55,5	74,1
OP50 + L4 worms + M9 mix (µl)	500	496,3	492,6	488,9	485,2	481,5	463	444,5	425,9

Apoptosis assay:

Exposure of L4 worm for 24 hours in a liquid medium

Calculations:

DMNA

40 m.M.: 555 m.M. * X = 40 m.M. * 500 µl

X = 40 m.M. * 500 µl / 555 m.M. = 36,0 µl.

60 m.M. 555 m.M. * X = 60 * 500 µl

X = 60 m.M. * 500 µl / 555 µl = 54,1 µl.

80 m.M. 555 m.M. * X = 80 m.M. * 500 µl

X = 80 m.M. * 500 µl / 555 m.M. = 72,1 µl.

100 m.M. 555 m.M. * X = 100 m.M. * 500 µl

X = 100 m.M. * 500 µl / 555 µl = 90,1 µl.

NDMA

40 m.M.: 675 m.M. * X = 40 m.M. * 500 µl

X = 40 m.M. * 500 µl / 675 m.M. = 29,6 µl.

60 m.M. 675 m.M. * X = 60 * 500 µl

X = 60 m.M. * 500 µl / 675 µl = 44,4 µl.

80 m.M. 675 m.M. * X = 80 m.M. * 500 µl

X = 80 m.M. * 500 µl / 675 m.M. = 59,3 µl.

100 m.M. 675 m.M. * X = 100 m.M. * 500 µl

X = 100 m.M. * 500 µl / 675 µl = 74,1 µl.

Heat shock and heat shock induced brood size analysis (same procedures for growing nematodes).

L1 worms were cultured on plates (3 cm) seeded with OP50 and three concentrations of DMNA and NDMA, in addition to a control:

DMNA:

1) 550 m.M. * X = 0,5 m.M. * 5000 µl

X = 4,5 µl NGM: 4995,5 µl to a total volume of 5000 µl

2) 550 m.M. * X = 5 m.M * 5000 µl

X = 45,5 µl NGM: 4963 µl to a total volume of 5000 µl

3) 550 m.M. * X = 20 m.M. * 5000 µl

X = 181, 8 µl NGM: 4818,2 µl to a total volume of 5000 µl

NDMA:

1) 675 m.M. * X = 0,5 m.M. * 5000 µl

X = 3,70 µl NGM: 4996,6 µl to a total volume of 5000 µl

2) 675 m.M. * X = 5 m.M. * 5000 µl

X = 37,0 µl NGM: 4963 µl

3) 675 m.M. * X = 20 m.M. * 5000 µl

X = 148,1 µl NGM: 4851 µl.

X.2. Chemicals

Lysogeny broth (LB)-medium:

0.5 g Casein peptone

0.25 g Yeast extract

Sodium chloride (NaCl)

Nematode growth medium (NGM) agar:

2.5 g of casein peptone

17 g of bacteriological agar

3 g of NaCl.

M9 medium:

6 g of Na_2HPO_4

3 g of KH_2PO_4

5 g of NaCl

0.25 g of $MgSO_4 * 7H_2O$

X.2. Raw data

Acute test

DMNA

Concentrations m.M.	Survival rate %
0 m.M.	100
99,9 m.M.	100
144 m.M.	100
222 m.M.	100
333 m.M.	100
488 m.M.	88
744 m.M.	0
1050 m.M.	0

NDMA

Concentrations m.M.	Survival rate %
0 m.M.	100
5 m.M.	100
10 m.M.	100
15 m.M.	100
20 m.M.	100
25 m.M.	100
50 m.M.	100
75 m.M.	100
100 m.M.	100

L4 assay

Egg hatching rate

Dimethylnitramine	**0 m.M.**	**0,1 m.M.**	**0,5 m.M**	**1 m.M.**	**5 m.M.**	**10 m.M.**	**20 m.M.**	**40 m.M.**	**60 m.M.**	**80 m.M.**
Replicate 1	69,3	77,1	65,5	77,8	78,9	64,7	62,9	20	0	0
Replicate 2	100	96,9	94,1	100	53,8	74,85	66,7	0	0	0
Replicate 3	100	100	100	100	100	97,2	92,3	0	0	0
Sum	269,3	274	259,6	277,8	232,7	236,75	221,9	20	0	0
Average	89,77	91,333	86,5333	92,6	77,567	78,917	73,9667	6,6667	0	0
Standard deviation	14,47	10,144	15,0666	10,465	18,885	13,576	13,0561	10	0	0
SEM values	8,36	5,86	8,71	6,05	10,92	7,85	7,55	5,78	0	0
Deviation from control group %	0	1,7	3,68	3,11	13,6	12,1	17,6	92,6	100	100

Egg hatching rate

Dimethylnitrosamine	**0 m.M.**	**0,1 m.M.**	**0,5 m.M**	**1 m.M.**	**5 m.M.**	**10 m.M.**	**20 m.M.**	**40 m.M.**	**60 m.M.**	**80 m.M.**
Replicate 1	82,7	78,7	82,8	57,3	74,6	68,3	82,3	60,8	45,8	35,3
Replicate 2	100	100	100	84,6	64,3	100	80	87,5	90	64,3
Replicate 3	100	100	99,5	100	100	100	100	100	100	86,4
Sum	282,7	278,7	282,3	241,9	238,9	268,3	262,3	248,3	235,8	186
Average	94,23	92,9	94,1	80,633	79,633	89,433	87,4333	82,767	78,6	62
Standard deviation	8,155	10,041	7,99291	17,656	15,003	14,944	8,93545	16,35	23,5497	20,9248
SEM values	4,714	5,804	4,62018	10,206	8,6721	8,6379	5,165	9,4506	13,6125	12,0953
Deviation from control group %	0	2,19	0,05	22,3	24	7,9	11,2	18,83	25,68	45,5

L4 stage day 4

Dimethylnitramine	**0 m.M.**	**0,1 m.M.**	**0,5 m.M**	**1 m.M.**	**5 m.M.**	**10 m.M.**	**20 m.M.**	**40 m.M.**	**60 m.M.**	**80 m.M.**
Replicate 1	100	100	100	100	100	100	93,2	0	0	0
Replicate 2	100	100	100	100	100	100	100	0	0	0
Replicate 3	100	100	100	100	100	100	0	0	0	0
Sum	300	300	300	300	300	300	193,2	0	0	0
Average	100	100	100	100	100	100	64,4	0	0	0
Standard deviation	0	0	0	0	0	0	45,6222	0	0	0
SEM values	0	0	0	0	0	0	26,3712	0	0	0
Deviation from control group %	0	0	0	0	0	0	100	100	100	100

L4 stage day 4

Dimethylnitrosamine	0 m.M.	0,1 m.M.	0,5 m.M	1 m.M.	5 m.M.	10 m.M.	20 m.M.	40 m.M.	60 m.M.	80 m.M.
Replicate 1	100	100	100	100	100	100	100	100	100	100
Replicate 2	100	100	100	100	100	100	100	0	0	0
Replicate 3	100	100	100	100	100	100	96,3	87,5	74,2	61,7
Sum	300	300	300	300	300	300	296,3	187,5	174,2	161,7
Average	100	100	100	100	100	100	98,8	62,5	58	53,9
Standard deviation	0	0	0	0	0	0	0	44,488	42,3888	41,1957
SEM values	0	0	0	0	0	0	0	25,716	24,5022	23,8126
Deviation from control group %	0	0	0	0	0	0	0	50	50	50

Gravid worms day 5

Dimethylnitramine	0 m.M.	0,1 m.M.	0,5 m.M	1 m.M.	5 m.M.	10 m.M.	20 m.M.	40 m.M.	60 m.M.	80 m.M.
Replicate 1	100	100	100	100	90	51,8	0	0	0	0
Replicate 2	96,5	100	91,6	84,8	76,9	20,5	0	0	0	0
Replicate 3	99,3	99,1	92,6	93	88,7	5,3	0	0	0	0
Sum	295,8	299,1	284,2	277,8	255,6	77,6	0	0	0	0
Average	98,6	99,7	94,7333	92,6	85,2	25,867	0	0	0	0
Standard deviation	1,512	0,4243	3,74641	3,5716	5,8929	19,359	0	0	0	0
SEM values	0,874	0,2452	2,16555	2,0645	3,4063	11,19	0	0	0	0
Deviation from control group %	0	1,11	4	6,1	13,6	73,77	100	100	100	100

Gravid worms day 5

Dimethylnitrosamine	0 m.M.	0,1 m.M.	0,5 m.M	1 m.M.	5 m.M.	10 m.M.	20 m.M.	40 m.M.	60 m.M.	80 m.M.
Replicate 1	100	100	95,3	89,9	96,8	94,7	61,4	8	0	0
Replicate 2	99,1	96,2	100	95,5	93,4	69,6	20,6	3	0	0
Replicate 3	100	100	100	95,8	94,4	96,8	83,05	8,6	2,1	0
Sum	299,1	296,2	295,3	281,2	284,6	261,1	165,05	19,6	2,1	0
Average	99,7	98,733	98,4333	93,733	94,867	87,033	55,0167	6,5333	0,7	0
Standard deviation	0,458	1,9349	2,39312	2,8629	1,7053	12,863	28,85	2,5702	0,80829	0
Deviation from control group %	0	1,46	1,9	6,88	4,47	7,5	51,81	94,48	100	100

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Length (m.M)/gravid Dimethylnitramine replicate 1

Worm	0	Gravid	1	Gravid	5	Gravid	7,5	Gravid	10	Gravid	15	Gravid	20	Gravid
1	1440	y	1200	y	560	n			1070	n	960	n	560	n
2	1000	y	1090	y	1130	y			930	n	700	n	410	n
3	1240	y	1070	y	660	n			760	n	600	n	310	n
4	1020	y	1070	y	1270	y			750	n	990	n	550	n
5	1090	y	1070	y	1270	y			920	n	860	n	510	n
6	1140	y	1350	y	1030	y			810	n	1030	n	640	n

7	1160	y	1130	y	1300	y		780	n	610	n	530	n
8	1030	y	1230	y	1210	y		610	n	700	n	450	n
9	1150	y	1170	y	1060	y		1000	y	930	n	400	n
10	1000	y	1270	y	1080	y		880	n	920	n	530	n
11	1160	y	980	n	1110	y		930	n	770	n	720	n
12	1010	y	1190	y	1100	y		940	n	970	n	540	n
13	1070	y	1030	y	1260	y		960	n	1070	y	420	n
14	1140	y	1140	y	1030	y		990	n	780	n	480	n
15	1270	y	1270	y	1090	y		600	n	940	n	720	n
16	1220	y	1330	y	720	n		930	y	850	n	680	n
17	1100	y	830	n	1170	y		750	n	640	n	500	n
18	1190	y	1310	y	1050	y		1190	y	950	n	390	n
19	1220	y	1190	y	1020	y		1280	y	800	n	840	n
20	1060	y	1270	y	1060	y		1010	y	870	n	600	n
21	1090	y	1210	y	1040	y		910	n	740	n	730	n
22	1220	y	1140	y	1210	y		1100	y	960	n	510	n
23	880	y	1170	y	740	n		990	y	780	n	680	n
24	1140	y	1270	y	1190	y		990	y	640	n	570	n
25	1050	y	1310	y	630	n		1080	y	990	n	610	n
26	1100	y	1230	y	1000	y		980	n	640	n	540	n
27	1170	y	1110	yy	1210	y		1010	y	880	n	810	n
28	1170	y	1100	y	740	n		970	n	900	n	710	n
29	1160	y	1240	y	1030	y		640	n	910	n	600	n
30	1220	y	1280	y				970	n	860	n	590	n
	33910		35250		29970			27730		25240		17130	
Average m.m.	1130,33		1175		1033			924,3		841,3		571	
Gravid %	100		93,3		79,3			33,3		3,3		0	
Standard deviation	103,778		113,1		203,1			155,4		131,9		125,3	
Deviation from control (decrease %)			3,5		-8,6			-18,2		-25,6		-50,5	

Dimethylnitramine replicate 2

Worm	0	Gravid	1	Gravid	5	Gravid	7,5	Gravid	10	Gravid	15	Gravid	20	Gravid
1	990	y	1320	y	1100	y	1060	y	990	n	1000	n	440	n
2	1370	y	1140	y	1010	n	970	n	990	n	790	n	680	n
3	1020	y	1230	y	1130	y	1270	y	940	n	920	n	630	n
4	980	y	1210	y	960	n	1190	y	970	n	800	n	720	n
5	1120	y	1350	y	1120	y	1100	y	1000	n	630	n	640	n
6	1250	y	1230	y	1240	y	1120	y	1020	n	700	n	740	n
7	1380	y	1240	y	1150	y	1170	y	950	n	870	n	620	n
8	1000	y	1260	y	1160	y	1080	y	1100	n	880	n	540	n
9	1130	y	1100	y	1200	y	760	n	890	n	920	n	570	n
10	1340	y	1210	y	1070	y	1020	y	910	n	700	n	790	n
11	1150	y	1280	y	1050	y	890	n	960	n	690	n	590	n
12	1070	y	1180	y	1040	n	1050	y	990	n	870	n	750	n

	13	1110	y	1210	y	1220	y	1120	y	630	n	820	n	730	n			
	14	1440	y	1260	y	1230	y	1080	y	770	n	980	n	530	n			
	15	1210	y	1210	y	1100	y	1110	n	1020	n	780	n	670	n			
	16	1340	y	1290	y	930	n	1020	y	1090	n	460	n	640	n			
	17	1180	y	1030	y	1100	y	1060	y	1030	n	780	n	750	n			
	18	1110	y	1160	y	1140	y	1040	n	1020	n	770	n	830	n			
	19	1290	y	1290	y	1330	y	1060	n	940	n	890	n	590	n			
	20	1320	y	1240	y	1190	y	1000	n	930	n	870	n	740	n			
	21	1200	y	1210	y	1110	y	1060	y	850	n	950	n	750	n			
	22	1230	y	1180	y	1070	y	1160	y	880	n	770	n	840	n			
	23	1300	y	1200	y	950	n	1190	y	750	n	780	n	960	n			
	24	1120	y	1310	y	1110	y	1230	y	970	n	650	n	610	n			
	25	1090	y	1290	y	1000	y	890	n	1070	n	950	n	680	n			
	26	1250	y	1150	y	1210	y	1180	y	870	n	930	n	620	n			
	27	1430	y	1370	y	1280	y	1050	n	1140	n	770	n	810	n			
	28	1380	y	1230	y	1100	y	760	n	950	n	780	n	660	n			
	29	1350	y	940	y	1400	y	1340	y	830	n	780	n	930	n			
	30	1300	y	1230	y	1360	y	940	n	880	n	810	n	620	n			
		36450		36550		34060		31970		28330		24290		20670				
Average		1215		1218		1135		1066		944,3		809,7		689				
Standard deviation		133,959		87,18		114,4		129,2		106,8		114,6		114,2				
Gravid %			100		100		86,7		66,7		0		0		0			
Deviation from control in %				0,2		-6,6		-12,3		-22,3		-33,4		-43,3				

Worm		0	Gravid	1	Gravid	5	Gravid	7,5	Gravid	10	Gravid	15	Gravid	20	Gravid
	1	1510	y	1470	y	1420	y	1310	y	1260	y	1040	n	1000	n
	2	1330	y	1470	y	1350	y	1280	y	1360	y	1200	y	1380	y
	3	1430	y	1460	y	1310	y	1380	y	1360	y	1290	y	1390	y
	4	1400	y	1410	y	1310	y	1360	y	1240	y	1210	y	890	n
	5	1260	y	1480	y	1250	y	1280	y	1210	y	1230	y	1270	n
	6	1460	y	1490	y	1360	y	1300	y	1310	y	1270	y	1280	y
	7	1530	y	1440	y	1440	y	1450	y	1390	y	1240	y	1150	n
	8	1460	y	1510	y	1350	y	1290	y	1220	y	1250	y	1060	n
	9	1480	y	1550	y	1440	y	1500	y	1200	y	1290	y	1070	n
	10	1330	y	1420	y	1330	y	1520	y	1310	y	1140	y	860	n
	11	1500	y	1460	y	1310	y	1370	y	1110	y	1360	y	740	n
	12	1630	y	1540	y	1350	y	1440	y	1340	y	1320	y	1080	n
	13	1640	y	1560	y	1360	y	1410	y	1310	y	1270	y	1200	y
	14	1600	y	1440	y	1250	y	1180	y	1290	y	1280	y	1120	n
	15	1510	y	1670	y	1320	y	1380	y	1250	y	1330	y		
	16	1590	y	1230	y	1380	y	1430	y	1380	y	1260	y		
	17	1560	y	1490	y	1330	y	1240	y	1230	y	1370	y		
	18	1580	y	1480	y	1230	y	1430	y	1320	y	1330	y		
	19	1370	y	1570	y	1260	y	1450	y	1460	y	1340	y		
	20	1540	y	1590	y	1400	y	1500	y	1400	y	750	n		

21	1450	y	1660	y	1380	y	1350	y	1340	y	1390	y		
22	1550	y	1510	y	1330	y	1320	n	1400	y	1240	y		
23	1350	y	1300	y	1310	y	1390	y	1350	y	1320	y		
24	1260	y	1270	y	1370	y			1440	y	1110	y		
25	1510	y	1250	y	1420	y			1430	y	1350	y		
26	1650	y	1360	y	1290	y			1370	y	1380	y		
27	1230	y	1160	y	1360	y			1470	y	1380	y		
28			1300	n	1220	y			1320	y	1310	y		
29			1310	y	1300	y			1330	y	1450	y		
30			1380	y	860	n			1410	y				
	39710		43230		39590		31560		39810		36700		15490	
Average	1470,74		1441		1320		1372		1327		1266		1106	
Standard deviation														
Deviation from control in %			-2		-10,3		-6,7		-9,8		-14		-24,8	
Gravid %		100		96,7		96,7		96,7		100		93,1	28,6	

DMNA m.M.	0	1	5	7,5	10	15	20
Size deviation compared to control (%)							
Replicate 1		3,5	-8,6		-18,2	-25,6	-50,5
Replicate 2		0,2	-6,6	-12,3	-22,3	-33,4	-43,3
Replicate 3		-2	-10,3	-6,7	-9,8	-14	-24,8
		1,7	-25,5	-19	-50,3	-73	-118,6
Average		0,567	-8,5	-9,5	-16,77	-24,33	-39,53

Length (mM)/gravid — Dimethylnitrosamine replicate 1

Worm	0	Gravid	1	Gravid	5	Gravid	7,5	Gravid	10	Gravid	15	Gravid	20	Gravid
1	1440	y	1240	y	1150	y	1240	y	1210	y	780	n	890	n
2	1000	y	1170	y	1090	y	990	y	1030	y	1080	y	570	n
3	1240	y	1160	y	1120	y	1020	y	1110	y	1020	y	670	n
4	1020	y	1320	y	970	y	1190	y	1070	y	1070	y	780	n
5	1090	y	1080	y	1230	y	1080	y	1180	y	1010	n	860	n
6	1140	y	1070	y	1090	y	1100	y	1190	y	1000	y	720	n
7	1160	y	1070	y	980	y	810	n	1080	y	1090	y	630	n
8	1030	y	1090	y	1070	y	1190	y	900	n	970	y	810	n
9	1150	y	1320	y	1260	y	940	y	880	n	770	n	510	n
10	1000	y	1160	y	1130	y	1100	y	1190	y	1110	y	570	n
11	1160	y	1330	y	1150	y	1260	y	1200	y	1010	y	590	n
12	1010	y	1210	y	1170	y	1040	y	1070	y	1210	y	730	n
13	1070	y	1090	y	1310	y	1230	y	1220	y	1220	y	880	n
14	1140	y	1070	y	1170	y	920	n	1200	y	1190	y	620	n
15	1270	y	1240	y	1210	y	1250	y	1150	y	1190	y	820	n
16	1220	y	1110	y	1240	y	1090	y	1100	y	870	n	700	n
17	1100	y	1120	y	1230	y	1250	y	1050	y	1100	y	500	n

18	1190	y	1180	y	1150	y	1180	y	1220	y	1120	y	640	n
19	1220	y	1160	y	1150	y	1090	y	1180	y	1120	y	830	n
20	1060	y	1120	y	1180	y	1070	y	1000	y	1150	y	910	n
21	1090	y	1060	y	1110	y	1100	y	1130	y	1170	y	900	n
22	1220	y	1210	y	1200	y	1200	y	1120	y	1090	y	930	n
23	880	y	1030	y	1120	y	1130	y	870	n	1010	y	570	n
24	1140	y	1180	y	1220	y	1120	y	1110	y	1120	y	680	n
25	1050	y	1130	y	1200	y	1100	y	700	n	910	n	830	n
26	1100	y	1120	y	1020	y	1090	y	1140	y	1130	y	890	n
27	1170	y	1160	y	1090	y	1110	y	1220	y	1150	y	660	n
28	1170	y	1050	y	1150	y	1020	y	1040	y	1180	y	810	n
29	1160	y	1060	y	1100	y	1150	y	1040	y	990	n		
30	1220	y	1210	y	1130	y	1010	y	1170	y	1100	y		
	33910		34520		34390		33070		32770		31930		20500	
Average	1130,333		1151		1146		1102		1092		1064		732,1	
Standard deviation	103,7781		80,91		76,48		103,9		121,3		114,7		130,3	
Gravid %	100		100		100		93,3		86,7		80		0	
Deviation of control %			1,8		1,4		-2,5		-3,4		-5,8		-35,2	

Length (m.M)/gravid Dimethylnitrosamine replicate 2

Worm	0	Gravid	1	Gravid	5	Gravid	7,5	Gravid	10	Gravid	15	Gravid	20	Gravid
1	1370	y	1150	y	1170	y	1320	y	1220	y	1210	y	1160	y
2	1250	y	1250	y	1250	y	1210	y	1350	y	1310	y	1270	y
3	1320	y	1210	y	1220	y	1240	y	1110	y	1170	y	1350	y
4	1360	y	1240	y	1150	n	1230	y	1240	y	1110	y	1230	y
5	1500	y	1230	y	1260	y	1240	y	1280	y	1270	y	1390	y
6	1360	y	1380	y	1240	n	1250	y	1320	n	1320	n	1120	y
7	1340	y	1220	y	1210	n	1260	y	1240	y	1130	y	1340	n
8	1420	y	1260	y	1330	y	1190	y	1360	y	1290	y	1280	y
9	1440	y	1300	y	1290	y	900	y	1240	y	1270	y	1290	y
10	1420	y	1260	y	1360	y	1320	y	1190	y	1360	y	1300	y
11	1450	y	1350	y	1260	y	1280	y	1370	y	1350	y	1260	y
12	1440	y	1120	y	870	n	1070	y	1320	y	1360	y	1130	y
13	1280	y	1140	y	1360	y	1170	y	1230	y	1320	y	1260	n
14	1460	y	1320	y	1360	y	1240	y	1080	y	1260	y	1190	y
15	1410	y	1190	y	1010	n	1330	y	1260	y	1270	y	1310	y
16	1500	y	1160	y	1090	n	1250	y	1200	y	1170	y	1300	y
17	1450	y	1250	y	1150	n	1250	y	1360	y	1320	y	1330	y
18	1470	y	1130	y	1160	y	1330	y	1300	y	1250	y	1340	y
19	1580	y	1210	y	1030	y	1340	y	1210	y	1230	y	1330	y
20	1050	y	1280	y	1030	y	1050	y	1270	y	1320	y	1290	y
21	1470	y	1120	y	1140	y	1160	y	1110	y	1290	y	1350	y
22	1410	y	1330	y	1080	y	1210	y	1080	y	1320	y	1430	y
23	1510	y	1380	y	1150	y	1310	y	1240	y	1320	y	1320	y
24	1430	y	1240	y	1290	y	1350	y	1120	y	1200	y	1380	y

	25	1490	y		1270	y		1160	y		1130	y		1320	y		1410	y		990	y				
	26	1520	y		1280	y		1090	y		1200	y		1320	y		1310	y		1320	n				
	27	1470	y		1320	y		1120	y		1300	y		1150	y		1340	y		1420	y				
	28	1450	y		1280	y		1290	y		1260	y		1300	y		1310	y		1270	y				
	29	1310	y		1290	y					1181	y		1220	y		1290	y		1250	y				
	30	1370	y		1080	y					1000	y		1260	y		1360	y		1390	y				
		42300			37240			33120			36571			37270			38440			38590					
Average		1410			1241			1183			1219			1242			1281			1286					
Standard deviation		99,56572			77,62			117,2			103,3			83,09			70,27			93,33					
Gravid %				100			100			82,1			100			96,7			96,7			93,3		
Deviation of control %					-12			-17,3			-16,5			-12			-9,1			-8,8					

Replicate 3

Worm	0	Gravid	1	Gravid	5	Gravid	7,5	Gravid	10	Gravid	15	Gravid	20	Gravid
1	1400	y	1380	y	1390	y	1360	y	1410	y	1030	y	1310	y
2	1390	y	1350	y	1190	y	1280	y	1290	y	1470	y	1260	y
3	1360	y	1320	y	1380	y	1280	y	1350	y	1290	y	1270	y
4	1460	y	1340	y	1350	y	1390	y	1400	y	1380	y	1310	y
5	1460	y	1340	y	1330	y	1370	y	1330	y	740	y	1350	y
6	1400	y	1020	n	1420	y	1410	y	1400	y	1480	y	1250	y
7	1410	y	1310	y	1410	y	1340	y	1440	y	1440	y	1210	y
8	1350	y	1460	y	1430	y	1300	y	1350	y	1340	y	1260	y
9	1430	y	1390	y	1410	y	1330	y	1400	y	1320	y	1300	y
10	1450	y	1340	y	1240	y	1290	y	1390	y	1370	y	1310	y
11	1440	y	1400	y	1350	y	1420	y	1130	y	1470	y	1290	y
12	1360	y	1460	y	1360	y	420	y	1340	y	1460	y	1320	y
13	1440	y	1420	y	1470	y	1350	y	1440	y	1340	y	1390	y
14	1500	y	1410	y	1420	y	1310	y	1430	y	1430	y	1400	y
15	1440	y	1330	y	1420	y	1350	y	1440	y	1420	y	1360	y
16	1390	y	1460	y	1440	y	1300	n	1420	y	1350	y	1300	y
17	1310	y	1360	y	1360	y	1190	y	1410	y	1350	y	1220	y
18	1390	y	1400	y	1380	y	1430	y	1480	y	1430	y	1310	y
19	1390	y	1350	y	1430	y	1320	y	1430	y	1340	y	1250	y
20	1360	y	1360	y	1370	y	1420	y	1520	y	1360	y	1310	y
21	1410	y	1360	y	1410	y	1420	y	1450	y	1450	y	1330	y
22	1420	y	1510	y	1340	y	1320	y	1510	y	1420	y	1260	y
23	1440	y	1350	y	1440	y	1230	y	1520	y	1430	y	1290	y
24	1300	y	1370	y	1370	y	1370	y	920	n	1320	y	1220	y
25	1370	y	1430	y	1330	y	1400	y	540	y	1460	y	1410	y
26	1390	y	1440	y	1390	y	1310	y	1560	y	1490	y	1350	y
27	1440	y	1260	y	1350	y	1300	y	1480	y	1430	y	1390	y
28	1310	y	1440	y	1280	y	1400	y	1460	y	1340	y	1300	y
29	1410	y	1430	y	1010	y	1360	y	1460	y			1450	y
30	1440	y	1450	y	1410	y	1420	y					980	n
	42060		41240		40880		39390		39700		38150		38960	
Average size	1402		1375		1363		1313		1369		1363		1299	

Standard deviation								
Gravid %	100	97	100	97	96,7	100	96,7	
Deviation of control %	-1,9	-2,8	-6,3	-2,4	-2,8	-7,4		

NDMA m.M.	1	5	7,5	10	15	20
Size deviation compared to control (%)						
Replicate 1	1,8	1,4	-2,5	-3,4	-5,8	-35,2
Replicate 2	-12	-17,3	-17	-12	-9,1	-8,8
Replicate 3	-1,9	-2,8	-6,3	-2,4	-2,8	-7,4
	-12	-18,7	-25	-17,8	-18	-51,4
Average	-4	-6,233	-8,4	-5,933	-5,9	-17,13

Heat shock induced brood size

	Average	Standard deviation	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Control	181	32,04293994	199,5	199,5	199,5	125,5		
Control + HS	150,5	25,11473671	165	165	165	107		
DMNA 0,5 m.M. + HS	99	26,38749704	96	44	95	54		
DMNA 5 m.M. + HS	99	46,31954231	92	153	123	28	64	
DMNA 20 m.M. + HS	79,5	30,90711892	76	57	54	131	136	163
NDMA 0,5 m.M. + HS	85,3	25,72329683	44	102	84	111		
NDMA 5 m.M. + HS	94,8	36,06505234	63	156	82	78		
NDMA 20 m.M. + HS	41,3	18,75334637	60	12	55	38	65	114
DMNA -HS 0,5 m.M.	165	7	158			172		
DMNA - HS 5 m.M.	144,5	13,5	158			131		
DMNA - HS 20 m.M.	132	4	128			136		
NDMA - HS 0,5 m.M	120	17	137			103		
NDMA -HS 5 m.M.	156	13	143			169		
NDMA -HS 20 m.M.	111	28	83			139		

Heat shock:

Replicate 1 Survival rate

Hours	**Control**	**Control + HS**	**DMNA 0,5 mM**	**DMNA 5 mM**	**DMNA 20 mM**	**NDMA 0,5 mM**	**NDMA 5 mM**	**NDMA 20 mM**
1	100	100	100	100	100	100	100	100
2	100	100	100	100	100	100	100	100
3	100	80	80	100	100	100	100	90
4	100	60	70	90	100	80	100	80
5	100	20	10	90	80	40	70	80

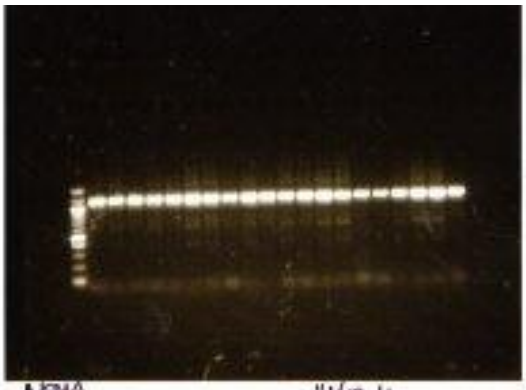
Replicate 2	Control	Control + HS	DMNA			NDMA		
			0,5	5	20	0,5	5	20
1 h		100	100	100	100	100	100	100
2 h		100	90	100	100	100	100	100
3 h		100	80	100	100	90	100	100
4 h		70	80	100	80	80	100	100
5 h		70	70	80	80	80	80	90
6 h		0	60	80	40	0	60	70

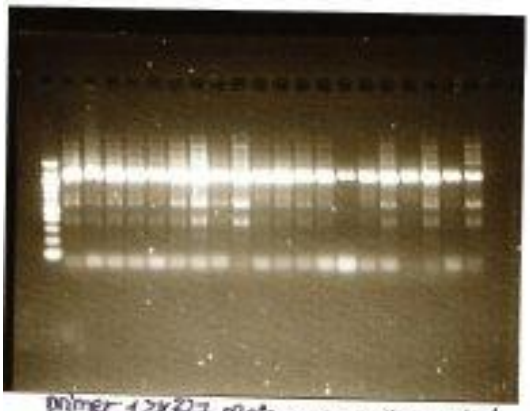
Apoptotic corpses:

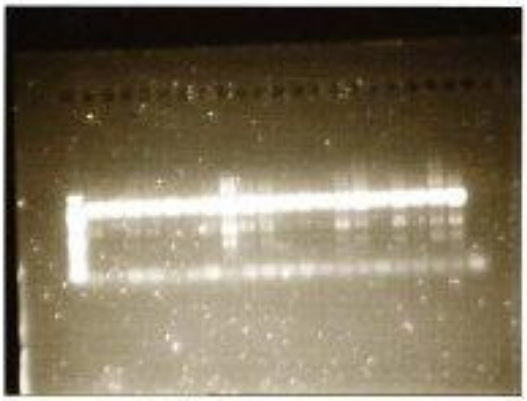
Individuals	Control	DMNA 40 mM	DMNA 60 mM	DMNA 100 mM	NDMA 40 mM	NDMA 60 mM	NDMA 80 mM	NDMA 100 mM	ENU 5 mM
1	8	8	16	8	7	7	12	20	9
2	9	10	14		10	8	8	8	11
3	8	12	23	6	8	10	7	8	11
4	9	11	15	7	7	11	6	7	10
5	5	8	5	9	4	8	11	19	23
6	9	6	11	5	7	7	17	10	11
7	5	6	10	10	11	12	7	9	16
8	2	9	13	8	7	7	14	9	20
9	0	5	9	12	9	11	12	10	10
10	5	12	9	5	7	8	6	10	13
11	2	9	15	7	7	4		9	12
12	5	14	28	5	8	14		9	9
13	0	5	9	13	7	10		12	21
14	5	11	12	11	3	11		8	10
15	3	5	10	9	5			11	22
Sum	75	131	199	115	107	128	100	159	208
Average	5	8,733333	13,26667	8,21429	7,13333	8,53333	10	10,6	13,8667
Standard deviation	3,0332	2,815828	5,638755	2,51222	1,99555	2,508	3,57771	3,702252	4,92432

X.4. Photos of PCR results

Primer 1:







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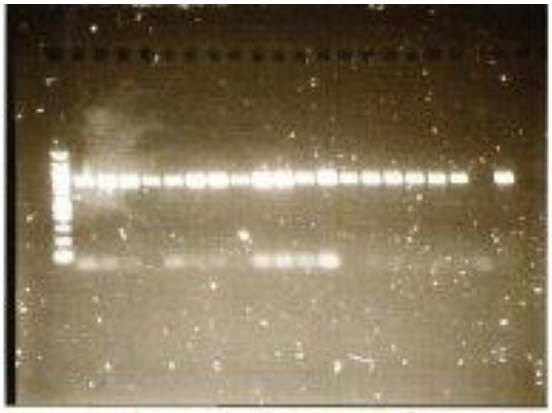
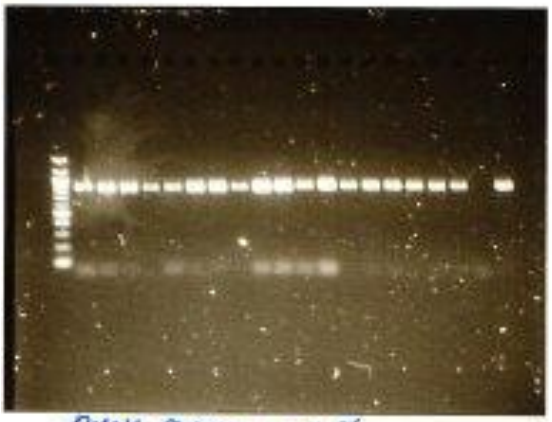


DNA primer 2 60.9 °C
15/12-11

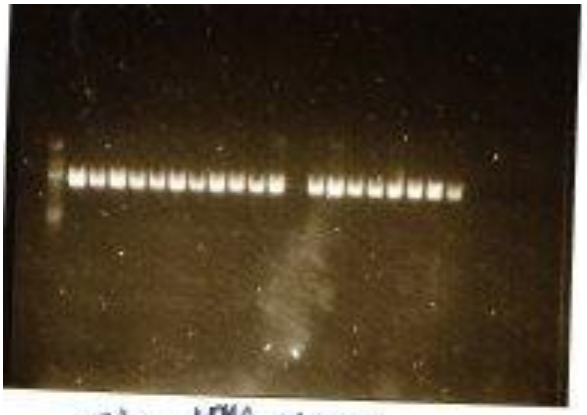
DNA primer 2 M106
16/12-11 60.9°C



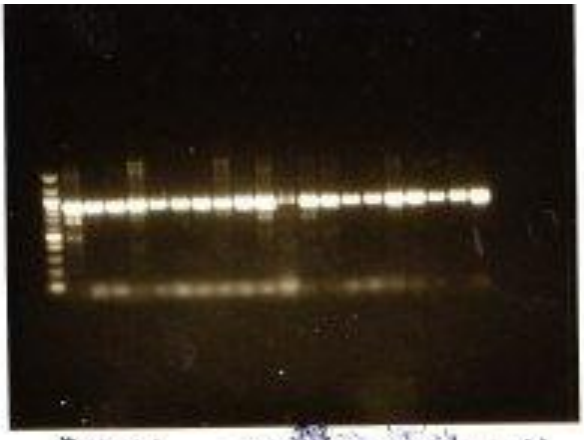
DNA primer 2 60.9 °C
15/12-11

Untreated primer 2 M106
60.9°C 16/12-11

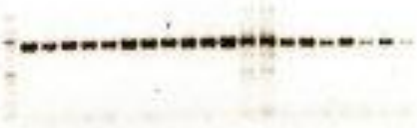
Primer 3:

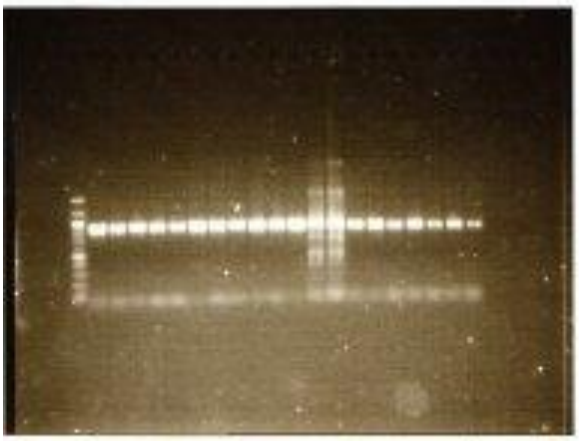


17/11 MMA primer3



DMNA -primer3 17/11-11
+nr 12, nr 13 in lane 12





Primer ⑤ Untreated 17/11-11

X.5. DNA sequencing results

Fasta file Control:

Ctrl

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4R              TCGTCATC---GTCTTCTTCTTTTCCATCTTTAGGCTCTCCGCCACTGACAGAGACAGGC 146
5R              ACTTG--C---GTCT----------CACTTTT-----------CACT------------- 66
7R              TCTTATTCTTGGTTTCCAT------AATTTGTAGAC----CGCCAATTTCAGAGTCTGAA 166
10R             A-------------------------ATATTC----------CCGATAGAGAAGGATATC 104
13R             A-------------------------ACA-------------CAGTAAACA-------C 66
                                  *               *  *

4R              ATCTGCTGATGAGAGTGTGCCAGGACGTGTGAGACGCAGACTCATTGCCATCGGAACACA 206
5R              ---------TGTGTGTGTGTTCCGATG--------GCA---------------------- 87
7R              GTAGATTGTCGAAAGTGAGCAAGAACGCAGTTACGGTGAGTCA--TCCAATCGGAACACA 224
10R             ATGGAACAATGAG-GTTCATCAGAGTT-ATTTGTCAAAAATTT--------------GCA 148
13R             ATAGA----TGAC-ATGCGGTAAA----------CGAAAAT------------------- 92
                     *    *

4R              CATACAAGTGACCCGAGAGACGCAGACACAGTAGACACATTGATTTACATTCGGGGAAGC 266
5R              ------------------------------------------------------------
7R              CAGACAAGAGGCCCGAGATACGCAGACACAGTA-ACACATTGATT-ACATTCGGTTAGTC 282
10R             AAAGAAAACGCTCAACGATAG-------------------------------------- 169
13R             AACGTAATTGC---------------------------------------------- 103


4R              ATTTCAAAAATTATC--- 281
5R              ------------------
7R              --TTGAATGTTTATTTTT 298
10R             ------------------
13R             ------------------
```

Fasta file DMNA:

DNMA

>13F

CCCCTATATTGCCAATAAAGTTATTCTGCGCAAATCCAGATTCTCGTAGGCGCCACGAAA
TTTCGTCAGCTGAAAAATCGGGGTGTGCGACTTGTGGCTATTTTA

>1R

CGGATAATTAGTCCTGGCACCGCGCGTGCTTTCGATTTATAAAAAGAATTGAGAACAATG
GAAACGCGCGATGCCTAACATCTGCGAGACCGAGAATCTATCGTTGAGTGTTTTCTTGTG
GAGATTCGTGACAAATAAATCTGATGAACCCCAGTGTTCCATGATTTCCTTCTCCTTCGG
GAATATTCTTTATAAGGCTCAACTAGCACATTCCGAACTCTCTGTATGTTTGCCCTTGCT
GGGTTTCTTTTTTTTACAGCTCTTTCTTTTTTATCGAAACTCCGTCTAGTTCCACTATTT
ACTTTTTTTTGAGCCGAGGGGAGTGCCTGCCAAAAAA

>10R

CAA
TGTGTAACGCGCCGATGCCTAGTATCTGCAANACCGAGAATCTATCGTTGTGCGTTTTCT
TGTGCAAATTCGTGACNNANNAATCTGATGAACCTCAGNGTTCCATGATTTCTCTCTCCT
TCGTTGAATATTCCTTTTTAAGGCTCAACTAGCACATNCCGAACTCTTAGTANGTTTGCC

```
1R              CGGATAATTAGTCCTGGCACCGCGCGTGCTTTCGATTTATAAAAAGAATTGAGAACAATG 60
10R             ------------------------------------------------------CAATG 5
                                                                       *****

1R              -GAAACGCGC-GATGCCTAACATCTGCGAGACCGAGAATCTATCGTTGAGTGTTTTCTTG 118
10R             TGTAACGCGCCGATGCCTAGTATCTGCAANACCGAGAATCTATCGTTGTGCGTTTTCTTG 65
                 * ******* ********  ****** * ****************** * *********

1R              TGGAGATTCGTGACAAATAAATCTGATGAACCCCAGTGTTCCATGATTTCCTTCTCCTTC 178
10R             TGCAAATTCGTGACNNANNAATCTGATGAACCTCAGNGTTCCATGATTTCTCTCTCCTTC 125
                ** * *********  *  ************* *** *************  ********

1R              GG-GAATATTC-TTTATAAGGCTCAACTAGCACATTCCGAACTCTCTGTATGTTTGCCCT 236
10R             GTTGAATATTCCTTTTTAAGGCTCAACTAGCACATNCCGAACTCTTAGTANGTTTGCC-- 183
```

```
                *  ******** *** ****************** *********  *** *******

1R              TGCTGGGTTTCTTTTTTTTACAGCTCTTTCTTTTTTATCGAAACTCCGTCTAGTTCCACT 296
10R             ------------------------------------------------------------


1R              ATTTACTTTTTTTTGAGCCGAGGGGAGTGCCTGCCAAAAAA 337
10R             ----------------------------------------
```

Fasta file NDMA:

NDMA fasta

>1
CCATAAATTGCTATAAAAGAATTCGGCGCAAATGAGATTCTCGTAGGCGCCACGAAAATT
TCGTCAGATGAAAAAACGGGGTGTGCGACTTGCGGCTTTTTTA
>2
GCCCCAAATTGCCATTGAAGTATTCGGCGCAACTGAGATTCTCGTAGTCGCGACGAAATT
TTCGTCAGATGAAAAAACGGGGTGTCCGACTTGTGGCGAACTTTA
> 3
GCATAATTGCTAGAATTTTTCGGCGCACTCAGATCTCGGAGGCCCACGAAATTTCGTCAG
CTGAAAAAACGGGGTGTCCGACGTGGGGCCTTTTTA
>4
CAAAATTGAAAAAAATTTTTTTTTGCAGAAATGAGATTTTGGAGGGGCGACGAATTTTCGT
CAAATGAAAAAACGCGTTGTGCGACTTGAGGAAACTTATTGTTTTAACTCGTGGCTCTCA
CAATTTTTTTAAAAATTTATTCAAGTTAGTTTTTTTTTCAATA
>5
AAAAGTTCATCAAAGGTTATTCGGCGCCACTCAGATCTCGTTGTCGACACGAAATTTCGT
TGAAGTTTTTCGGGGTGTCCGACTTGGGGCGAACTTTA
>7
CACCAAAACTTCCCAAGAATATATTCTGGCGCACATCGAGATTCTCGTAGGCGCGACGAA
ATTTCGTCAAATGAAAAAACGGGGTGTGCGACTTGTGGCGAACTTTA
>8
ACCAAAATTACCACAATATTTTTTCCGGCACCCCTCAAGATTCTCGGAGGCGCGACGAAA
TTTCGTCAAATGAAATAACGGCGTGTGCGACTTGTGGCGAACTTTATT
>9
CCCATAATGTCAATGACGTTTTCGGCGCACCTCCAGATCTCGTAGTCGAGACGAAATTTC
GTCAGATGAAAAAACGGGGTGTCCGACTTGTGGCGAACTTTA
>10
GCATAATTTCAATGACGTTTTTTGCGCACCTGAGATTTTCCGAGTCCCACGAATTTTCTT
CAAAATAAAAATTGGGGTGGGCTTGTGGGACTTTCTTTA
>11
CCCAATTGCTTAGACTTTTTCTGCGCACCATGAGATCTGTAGGCGACACGAAATTTCTTT
GAATTTTTTCGGTGTGTCCGAGTTGCGGCTTTGATA
>13
CGGTACCAAGCTTGGNNNATNC
NGGTNNANANGTTTNCTGTGTCAATTGTTATCCCCTCACAATTCCACACGACCTACGAGC
CGGTNGCGTGGATTGTAAAGGCTGGGGTGCCTAATGAGTGAN
>15
GAACTGCTTATCATTCAAAAATTTTCTTGCGCCAATGAGATTCTGTAGGGGCGACGAATT
TTCGTCGATGAAAAAACGGGTTGTGCGACTTGTGGCGAACTTTA
>16
CCACAACCGCCCAACACTAATTTTCTTGCACACCTGAGATTCTTCGGAGTCGCGACGAAT
TTTCGTCAAAAGAAAAAACGGGGTGTCCGACTTGTGGCGAACTTT
>17
CCAATAGTCATACGAATTTTTCTTGCGCACACGAGATTCTCGGAGTCGCGACGAAATTTC
GTTGATGAAAAAACGGGGTGTGCGACTTGGGGCGAACTTAAAAG
>18

GCATAATTGGCTAAGAGTTATTCGGCGCACATCCAGATTCTCGTAGTCGAGACGAAAATT
TCGTCAGAAGAAAAAACGGGGTGTCCGACTTGTGGCGAACTTTA

>19

CAAAACTTTCCTAAAAAATTTTTTGGGCGCCCCTCCAGATTCTCGGAGGCGCGACGAATT
TTCGTCAAATGAAAAAACGGGGTGTGCGACTTGTGGCAAATTATA

```
9              -------------CCCATAAT---GTCAATGAC---GTTTTCGGCGCACCTCCAGAT-CT 40
18             -------------GCATAATT--GGCTAAGAG---TTATTCGGCGCACATCCAGATTCT 41
2              ------------GCCCCAAATT--GCCATTGAA---GTATTCGGCGCA-ACTGAGATTCT 42
5              --------------AAAAGTT--CATCAAAGG---TTATTCGGCGC-CACTCAGAT-CT 38
17             -------------CCAATAGT---CATACGAAT---TTTTCTTGCGCACAC-GAGATTCT 40
4              ---------------CAAAATT----GAAAAAAA-TTTTTTTTGCAGAAAT-GAGATTTT 39
15             -------GAACTGCTTATCATT----CAAAAA----TTTTCTTGCGCCAAT-GAGATTCT 44
7              ------------CACCAAAACTTCC-CAAGAATATATTCTGGCGCACATC--GAGATTCT 45
16             --------------CCACAACCGCC-CAACACTA-ATTTTCTTGCACACCT-GAGATTCT 43
8              -------------ACCAAAATTACCACAATATT---TTTTCCGGCACCCCTCAAGATTCT 44
19             --------------CAAAACTTTCCTAAAAAAT---TTTTTGGGCGCCCCTCCAGATTCT 43
1              --------------CCATAAATTGCTATAAAAG----AATTCGGCGCAA-ATGAGATTCT 41
3              --------------GCATAA-TTGCTA--GAAT----TTTTCGGCGCAC--TCAGAT-CT 36
11             ---------------CCCAA-TTGCTT-AGACT----TTTTCTGCGCACCATGAGAT--C 37
10             --------------GCATAATT---TCAATGACG---TTTTTTGCGCACCT-GAGATTTT 39
13             CGGTACCAAGCTTGGNNNATNCNGGTNNANANG----TTTNCTGTGTCAATTGTTATCCC 56
                                                  *   *          **

9              ----------CGTAGTCGAGACGAAA---TTTCGTCAGATGAAAAAA--CGGGGTGTCCG 85
18             ----------CGTAGTCGAGACGAAAA--TTTCGTCAGAAGAAAAAA--CGGGGTGTCCG 87
2              ----------CGTAGTCGCGACGAAAT--TTTCGTCAGATGAAAAAA--CGGGGTGTCCG 88
5              ----------CGTTGTCGACACGAAA---TTTCGT----TGAAGTTTTTCGGGGTGTCCG 81
17             ----------CGGAGTCGCGACGAAA---TTTCGTT-GATGAAAAAA--CGGGGTGTGCG 84
4              ----------GGAGGGGCGACGAAT---TTTCGTCAAATGAAAAAA--CGCGTTGTGCG 83
15             ----------GTAGGGGCGACGAAT---TTTCGTCGA-TGAAAAAA--CGGGTTGTGCG 87
7              ----------CGTAGGCGCGACGAAA---TTTCGTCAAATGAAAAAA--CGGGGTGTGCG 90
16             T---------CGGAGTCGCGACGAAT---TTTCGTCAAAAGAAAAAA--CGGGGTGTCCG 89
8              ----------CGGAGGCGCGACGAAA---TTTCGTCAAATGAAATAA--CGGCGTGTGCG 89
19             ----------CGGAGGCGCGACGAAT---TTTCGTCAAATGAAAAAA--CGGGGTGTGCG 88
1              ----------CGTAGGCGCCACGAAAA--TTTCGTCAGATGAAAAAA--CGGGGTGTGCG 87
3              ----------CGGAGGC-CCACGAAA---TTTCGTCAGCTGAAAAAA--CGGGGTGTCCG 80
11             ----------TGTAGGCGACACGAAA---TTTCTTTGAATTTTT--T--CGGTGTGTCCG 80
10             C---------CG-AGTC-CCACGAAT---TTTCTTCAAAATAAAAAT--TGGGGTG---G 80
13             CTCACAATTCCACACGACCTACGAGCCGGTNGCGTGGATTGTAAAGGC-TGGGGTGCCTA 115
                           ****     *  * *              *   **

9              ACTTG-----------------------TGGC----------------GAACTTTA---- 102
18             ACTTG-----------------------TGGC----------------GAACTTTA---- 104
2              ACTTG-----------------------TGGC----------------GAACTTTA---- 105
5              ACTTG-----------------------GGGC----------------GAACTTTA---- 98
17             ACTTG-----------------------GGGC----------------GAACTTAAAAG- 104
4              ACTTGAGGAAACTTATTGTTTTAACTCGTGGCTCTCACAATTTTTTTAAAAATTTATTCA 143
15             ACTTG-----------------------TGGC----------------GAACTTTA---- 104
7              ACTTG-----------------------TGGC----------------GAACTTTA---- 107
16             ACTTG-----------------------TGGC----------------GAACTTT----- 105
8              ACTTG-----------------------TGGC----------------GAACTTTATT-- 108
19             ACTTG-----------------------TGGC----------------AAATTATA---- 105
1              ACTTG-----------------------CGGC------------------TTTTTTA-- 103
3              ACGTG-----------------------GGGC------------------CTTTTTA-- 96
11             AGTTG-----------------------CGGC------------------TTTGATA-- 96
10             GCTTG-----------------------TGGG----------------ACTTTCTTTA 99
13             ATGAG-----------------------TGAN---------------------------- 124
                   *                       *

9              --------------------
18             --------------------
2              --------------------
5              --------------------
17             --------------------
4              AGTTAGTTTTTTTTTCAATA 163
15             --------------------
7              --------------------
16             --------------------
8              --------------------
19             --------------------
1              --------------------
3              --------------------
```

```
11            --------------------
10            --------------------
13            --------------------
```

>1R

```
GGTCAACAANN
NTCCGTGCTATGCCTAGTATTCGCNAGTTTCGAATCTATCGTTGAGCNTTTTCATTTGNA
AATTCTTGTGAATGAAAATTGATGAACCACAGTGTACAATGTTATCNCTCTTNACAGNAA
TTTATTCATTTTTAAGTCTCAACGAGAACATTCCGGATTCTTTGAAAGTTTGCCCCAGCA
CAGATACNTTTGACTACAGCTCNTTCNNTTTTTTCNAACTCCGCCTT
```

>2R

```
ATTTTGGAGCATCATTAAAACTTCCCGAATGTCATCAATGTGTCAACTGTGTCTGCGTCT
CTCGGGTCACTTGTATGTGTGTTCCGATGGCAATGAGTCTGCGTCTCACACGTCCTGGCA
CACTCTCATCAGCAGATGCCTGTCTCTGTCAGTGGCGGAGAGCCTAAAGATGGAAAAGAA
GAAGACGATGACGACGATAAAAATTCTTTACCACACACTGCCCCACCAATTTTTTTTCGA
TGATATACGCAGTCAGTCAGTCAGTCATCGTTCTCTCTCTCTCTCTTTCTCTCAAATTTT
CGCTTCTTTTGTCTTCTGATGATCATAAAGGTACAAGAAACTTATGTGTGTGTGCACCAG
CA
```

>3R

```
CGGAATAATTAATTCAGCAAACGAAGGTTTTTCAATGTAATAAATGTGTGTGCAACTCTC
GAAACGCTTGAATGCCAAATCCGATGAGTTGGGTCTCCATCGTTGCGTGTTGTACGTTCT
CATTAGGTGAGGAATGACTCTGATTAACCACTTAGGCTATCATGGCCTTCACTAACACGA
TATCTACGATAAAAACTCTTTACCAGCCCATGCCGCATTCTTAGATTGTTTGCCCTTGCT
CGGTTACTTTTGATTACAGCTCTTTCTCTCTATCGCACTCCTCCTACTTCATCTACTTTC
GTCTTCTGGTGCCGATTGTAGTGACTGACTGACTAGT
```

>4R

```
AAAACCATCCTGGACCAACCTCCCCAAATGTAATTCAATGTGTCAACTGTGTCTGCGTCT
CTCGGGTCACTTGTATGTGTGTTCCGATGGCAATGAGTCTGCGTCTCACACGTCCTGGCA
CACTCTCATCAGCAGATGCCTGTCTCTGTCAGTGGCGGAGAGCCTAAAGATGGAAAAGAA
GAAGACGATGACGACGATAAAAATTCTTTACCACACACTGCCCCACCAATTTTTTTTCGA
TGATATACGCAGTCAGTCAGTCAGTCATCGTACTCTCTCTCTCTCTTTCTCTCAAATTTT
CGCTTCTTTTGTCTTCTGATGATCATAAAGGTTCAAGAAACTTATGTGTGTGTGCACCAG
CAGCAGTCACCACAAGAGCGAACTGTCATATTCAATGTGTCACCTGTCAGCTTTTGCTCC
CCCATCTTGGCTCAAGGAAGCACAAATACGTGCTTTTGGCCTCCGCCTCTCCACTTTTCT
AATACCTAATATTTATCCTCGCCTATAGTTTCTTTATAGTTACC
```

>9R

```
NATATCTGCAGA
NTTCCANCACACTGGCGTNCCNTANAGAGNATCCNANCTCGGTNCAAGCTTGGCNNNTCN
TGGTCATAGCTGTTTCCTGAGTAAAATTGTTATCCNCTCACAATTCCACACGACATACGA
GCCGGAANCATAGATTGTAACGCCTGGGNTGCCNAATGAGTGGTCTNNCTCANNTNAATT
GCNTTGCGCTCACTGCCCGNTNTCNAGTCNGTAAACCNGTCGTGCC
```

>10R

```
AGAAGATCAGGCCAANNCNNNTACGAACCCTAAACCAAGGGAAGAGGACGATGAN
CCCGAAGAAGGAGAGATCGTGGGCGACGAANAATCGGCGTCGAAGCCATCNAAAGGAATA
GCGCCACAGTCTCATGCTCTGGAGCACTCATGGACCTTCTGGTTCGATAGCCCCGCAGCA
AAGTCCGCTAAGACTAANCAAGAGGATTGGGGGAGTTCCATTCGCCCGATCTATACCTTC
AGTACCGTCGAGGAGTTCTGGAGCATTTNCAATAATATACGTCATCCAAGCAAGTTGGCT
ATTGGCACGGACTTCCNTTGNTTCAAATNTAAAATTGAGCCAAAGTGGGAGGATCCTGTG
TGTGCTAGTGGAGGGAAGTGGACTGTAACTTTTCCTAAAGGGAAATCTGATACTTCTTGG
CTGTATACGTTGCTAGGAATGATAGGAGAACAGTTTGATCATGGAGATGAAATTTGTGGA
GCAGTTGNCAACGTCAGAAATAGGCAAGAAAAGATATCTATTTGGACGAAGATTGCAATN
AATGAAGCTGCTCANCTGAGCATTGGGAAACAGTGGAAGGGGTTTCTGGATTACNATGAA
ACTATAGGGTTCATATNTCTTGAGGATGCTNNGAGACATGAGAGAAAGTGCCAAAAATAA
ATAC
```

>12R

```
GGGAAAAGATTTTAAGCTCACCGAATGTCATCAATGTGTCAACTGTGTCTGCGTCTCTCG
GGTCACTTGTATGTGCGTTCCGATGGCAATGAGTCTGCGTCTCACACGTCCTGGCACACT
CTCATCAGCAGATGCCTGTCTCTGTCAGTGGCGGAGAGCCTAAAGATGGAAAAGAAGAAG
```

ACGATGACGACGATAAAAATTCTTTACCACACACTGCCCCACCAATTTTTTTTCGATGAT
ATACGCAGTCAGTCAGTCAGTCATCGTTCTCTCTCTCTCTCTTTCTCTCAAATTTCACGC
TTCTTTTGTCTTCTGATGATCATAAAGGCACAAGAAACTTATGTGTGTGTGCACCAGCAG
CAGCCACCACAAGAGCGAACTGTCATATTCAATGTGTCACCTGTCAGCTTTTGCTCCCCC
ATCTTGGCTCAAGGAAGCACAAATACGTGCTTTTGGCCTCCGCCTCTCCACTTTTCTAAT
ACCTAATATTTATCCTCGCCTATAGTTTCTTTATAGTAACCGGCTTGCTAGATTTAGACT
TTAGAGAACACGACAAATTTTTGATAGTTGCAAAATTTTGTGCGTCTTTAAAGAGTACTG
TAATTTCAAACTGCGGAATTTTATAGCTTTTCAATTAAAATATACTCTTTATTGAAAAAA
AAACTAACTTGAATAAATTTTTAAAAAAATTGTGAGACCCACGAGTTTAAAAACTATAAT
ACTCTTTAAAGGCGCACAACACATTTTTTTCATTTGACGAAAATTCCTCGCCCCTCCAAA
ACCTCATTTTCTACAAAAAAAATTTTTTTTGCAATTTTTTCCGTGTTTTCCGACTTGTGG
CCAAAACTTTTTAAAAAAGC

>14R

GGAAACTTATTATCCTATCATCATGACTTGATAACGAATGTAATATATGTTGATTTAGTT
TAAACTGCGCATCTGAATACGCTCTTTTTAGGAGACACTATCATTGTATTTTTGGAAAAT
TCAGACTATCAAGTTGAAACTCTGAAAAAACGCCATCTTCATTTGAAAAGGGCCCATAAT
TAGGAGCGTATTTTTACGCAAAGTGTTTGCTTTAATCATATAACTTACTTTGATTAACTT
CGATTGATTTTTTTATGATCATAGATATCTCTTACGATTGGAGTGACTGACTGAC

>15R

GGGTAAAGTAATTTTAGGCTACCAGAACGACTTCAAGATGATAACTGTGTCTGCATCTCT
CGAGTCGCTTGATGCCTGTACCGATGACATTTAGACTCCATCGTTCCGTCCTGAAACACT
CTCAATTGGAGATGACTGACTCTGATTATCCACTATGCCTATGTTGTCCTACACCAAGAC
GATGTCTACGATAAAAAGTCTTAACCAGCACCTGCCCCACTCGCAGTTAGTTTGCCCCTG
CTCGGTTTCATTTTATTACAGCTTCTTCTCTTTATCGCTTTCCTCGTACTTCCTCTTCTT
ATGTCTTCTGATGACGATTGGAGTGCCTGAAAGACATG

>16R

GGGGTAATGTAGTCTGGCAGCAGCGCGAGCTTTCGATTTAGAAAGAAAGTTGAGATCAAT
GTATCCGCGCCGATGCCTAGTATCTGCAAGTTTCGAATCTATCGTTGTGCGTTTTCTTTT
GGAAATTCGTGTAAAATGAATCTGATGAACCTCTTTGGTCCATGATTTCTCTCTCTTTCG
AATTTATTCTTTTTCAGGCTCAACTAGCACATTCCGAACTCTTTGAATGTTTGCCCTTGC
TGGGTTTCTTTTGATTACAGCTCTTTCTTTTTATCGAACTCCTTCTTCTTACTCGATTTA
CATTTTTTTGAGCCGATTGGAGTGACTGAAAAAACA

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2R              ------ATTTTGGAGCATCATTAAAACTTCCCGAATGTCAT-------CAATGTGTCAAC 47
4R              AAAACCATCCTGGACCAAC-------CTCCCCAAATGTAATT------CAATGTGTCAAC 47
12R             ----------GGGAAAAGATTTTAAGCTCACCGAATGTCAT-------CAATGTGTCAAC 43
1R              -------------GGT----------------CAACAANNN------------------ 12
16R             -----------GGGGTAATGT----AGTCTGGCAGCAGCGCGAGCTTTCGATTTAGAAAG 45
3R              ----------CGGAATAATTA----ATTCAGCAAACGAAGGTT--TTTCAATGTAATAAA 44
15R             -------GGGTAAAGTAATT------TTAGGCTACCAGAACGA--CTTCAAGATGATAAC 45
14R             -------------GGAAACTT----ATTATCCTATCATCATGA--------CTTGATAA- 34
9R              -------------NATATCT--------------GCAGANT----------TCCANCA-- 21


2R              TGTGTCTGCGTCTCTCGGGTCACTTGTATGTGT-GTTCCGATGGCAATGAGTCTGCGTCT 106
4R              TGTGTCTGCGTCTCTCGGGTCACTTGTATGTGT-GTTCCGATGGCAATGAGTCTGCGTCT 106
12R             TGTGTCTGCGTCTCTCGGGTCACTTGTATGTGC-GTTCCGATGGCAATGAGTCTGCGTCT 102
1R              ------------------TCCGTGCT-ATGCCTAGTATTCGCNA-GTTTCGAATCTATCG 52
16R             AAAGTTGAGATCAATGTATCCGCGCCGATGCCTAGTATCTGCAA-GTTTCGAATCTATCG 104
3R              TGTGTGTGCAACTCTCGAAACGCTTGAATGCCA-AATCCGATGA-GTTGGGTCTCCATCG 102
15R             TGTGTCTGCATCTCTCGAGTCGCTTGA-TGCCT-GTACCGATGACATTTAGACTCCATCG 103
14R             --------------------CGAATG--TAATATATGTTGATTTAGTTTAAACTGCGCAT 72
9R              --------------------CACTGGCGTNCCN--TANAGAGNA--TCCNANCTCGGTN- 56
                                    *       *                       *

2R              CACACGTCCTGGCACACTCTCATCAGCAGATGC-------CTGTCTCTGTCAGTGG--CG 157
4R              CACACGTCCTGGCACACTCTCATCAGCAGATGC-------CTGTCTCTGTCAGTGG--CG 157
12R             CACACGTCCTGGCACACTCTCATCAGCAGATGC-------CTGTCTCTGTCAGTGG--CG 153
1R              T-TGAG--------CNTTTTCATTTGNAAATTCTTGTGAATGAAAATT---GATGAACCA 100
16R             T-TGTG--------CGTTTTCTTTTGGAAATTCGTGTAAAATGAATCT---GATGAACCT 152
3R              T-TGCGTGTTGTA-CGTTCTCATTAGGTGAG-------GAATGACTCT---GATTAACCA 150
15R             T-TCCGTCCTGAAACACTCTCAATTGGAGAT-------GACTGACTCT---GATTATCCA 152
14R             C-TGAAT------ACGCTCTTTTTAGGAGACACTATC--ATTGTATTTTTGGAAAATTCA 123
9R              --CAAGCT------TGGCNNNTCNTGGTCAT-------AGCTGTTTCC------------ 89
                             *   *
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2R              GAGAGCCTAAAGATGGAAAAGAAGAAGA-CGATGACGACGATAAAAATTCTTTACCA-CA 215
4R              GAGAGCCTAAAGATGGAAAAGAAGAAGA-CGATGACGACGATAAAAATTCTTTACCA-CA 215
12R             GAGAGCCTAAAGATGGAAAAGAAGAAGA-CGATGACGACGATAAAAATTCTTTACCA-CA 211
1R              CAGTGTACAATGTTATCNCTCTTNACAG-NAATTTATTCATTTTTAAGTCTCAACGAGAA 159
16R             CTTTGGTCCATGATTTCTCTCTCTTTCG-AA-TTTATTC-TTTTTCAGGCTCAACTAGCA 209
3R              CTTAGG-CTATCATGGCCTTCACTAACA-CGATATCTACGATAAAAACTCTTTACCAGCC 208
15R             CTATGC-CTATGTTGTCCTACACCAAGA-CGATGTCTACGATAAAAAGTCTTAACCAGCA 210
14R             GACTAT--CAAGTTGAAACTCTGAAAAAACGCCATCTTCATTTGAAAAG-------GGCC 174
9R              ---TGAGTAAAATTGTTATCCNCTCACA--ATTCCACACGACATACGAGCCGGA--ANCA 142
                         *   *                       *

2R              CACTGCCCCACCAATTTTTTTTCGATGATATACGCAGTCAGTCAGTCAGTCATCGT--TC 273
4R              CACTGCCCCACCAATTTTTTTTCGATGATATACGCAGTCAGTCAGTCAGTCATCGT--AC 273
12R             CACTGCCCCACCAATTTTTTTTCGATGATATACGCAGTCAGTCAGTCAGTCATCGT--TC 269
1R              CA-TTCCGGA-------TTCTTTGAAAGTTTGCCC--CAGCACAGATACNTTTGAC--TA 207
16R             CA-TTCCGAA-------CTCTTTGAATGTTTGCCC--TTGCTGGGTTTCTTTTGAT--TA 257
3R              CA-TGCCGCA-------TTCTTAGATTGTTTGCCC--TTGCTCGGTTACTTTTGAT--TA 256
15R             CC-TGCCCCA-------CTCGCAGTTAGTTTGCCC--CTGCTCGGTTTCATTTTAT--TA 258
14R             CA-T----AA-------TTAGGAGCGTATTTTTAC--GCAAAGTGTTTGCTTTAATCATA 220
9R              TA------GA----------TTGTAACGCCTGGGNTGCCNAATGAGTGGTCTNNCT---- 182
                         *                   *

2R              TCTCTCTCTCTCTTTCTCT-CAAATTTT-CGCTTCTTTTGTCTTCTGATGATCATAAAGG 331
4R              TCTCTCTCTCTCTTTCTCT-CAAATTTT-CGCTTCTTTTGTCTTCTGATGATCATAAAGG 331
12R             TCTCTCTCTCTCTTTCTCT-CAAATTTCACGCTTCTTTTGTCTTCTGATGATCATAAAGG 328
1R              CAGCTCNTTCNNTTTT--TTCNAACTC--CGCCTT-------------------------- 238
16R             CAGCTCTTTCTTTTTA--T-CGAACTC--CTTCTTCTTACTCGATTTAC-ATTTTTTTGA 311
3R              CAGCTCTTTCTCTCTA--T-CGCACTC--CTCCTACTTCATCTACTTTC-GTCTTCTGGT 310
15R             CAGCTTCTTCTCTTTA--T-CGCTTTC--CTCGTACTTCCTCTTCTTAT-GTCTTCTGAT 312
14R             TAACTTACTTTGATTAACTTCG-ATTG--ATTTTTTATGATC-ATAGAT-ATCTCTT--- 272
9R              CANNTNAATTGCNTTG--CGCTCACTGC-CCGNTNTCNAGTCNGTAAAC----------- 228
                    *   *     *     *    *       *

2R              TACAAGAAACTTATGTGTGTGTGCACCAGCA----------------------------- 362
4R              TTCAAGAAACTTATGTGTGTGTGCACCAGCAGCAGTCACCACAAGAGCGAACTGTCATAT 391
12R             CACAAGAAACTTATGTGTGTGTGCACCAGCAGCAGCCACCACAAGAGCGAACTGTCATAT 388
1R              ------------------------------------------------------------
16R             GCCGA-----TTG-GAGTGACTGAAAAAACA----------------------------- 336
3R              GCCGA-----TTG-TAGTGACTGACTGACTAGT--------------------------- 337
15R             GACGA-----TTG-GAGTGCCTGAAAGACATG---------------------------- 338
14R             -ACGA-----TTG-GAGTGACTGACTGAC------------------------------- 294
9R              --CNG-----TCG----TGCC--------------------------------------- 238


2R              ------------------------------------------------------------
4R              TCAATGTGTCACCTGTCAGCTTTTGCTCCCCCATCTTGGCTCAAGGAAGCACAAATACGT 451
12R             TCAATGTGTCACCTGTCAGCTTTTGCTCCCCCATCTTGGCTCAAGGAAGCACAAATACGT 448
1R              ------------------------------------------------------------
16R             ------------------------------------------------------------
3R              ------------------------------------------------------------
15R             ------------------------------------------------------------
14R             ------------------------------------------------------------
9R              ------------------------------------------------------------


2R              ------------------------------------------------------------
4R              GCTTTTGGCCTCCGCCTCTCCACTTTTCTAATACCTAATATTTATCCTCGCCTATAGTTT 511
12R             GCTTTTGGCCTCCGCCTCTCCACTTTTCTAATACCTAATATTTATCCTCGCCTATAGTTT 508
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