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**FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN**  
*master in de biomedische wetenschappen: milieu en  
gezondheid*

**Masterproef**

Protective effect of Zn<sup>2+</sup> on heavy metal-induced reproductive toxicity

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*Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische  
wetenschappen , afstudeerrichting milieu en gezondheid*

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## Abbreviations

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-SH	Sulfhydryl
As	Arsenic
ATSDR	Agency for Toxic Substances and Disease Registry
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CaCl <sub>2</sub>	Calcium chloride
CAT	Catalase
CCVD	Cardiovascular and cerebrovascular disease
Cd	Cadmium
CdCl <sub>2</sub>	Cadmium chloride
CTR	Copper transporter
Cu	Copper
CuSO <sub>4</sub>	Copper sulfate
DMT	Divalent metal transporter
GSH	Glutathione
EGL	Egg-laying defective gene
GLD	Defective in germline development gene
GON	Abnormal gonad development gene
GSH	Glutathione
GST	Glutathione-S-transferase
H <sub>2</sub> O	Water
HSP	Heat shock protein
LC10	Lethal concentration that will kill 10% of the test subjects
LC50	Lethal concentration that will kill 50% of the test subjects
LC90	Lethal concentration that will kill 90% of the test subjects
K <sub>2</sub> HPO <sub>4</sub>	Dipotassium phosphate
KH <sub>2</sub> PO <sub>4</sub>	Monopotassium phosphate
KPO <sub>4</sub>	Potassium phosphate
Mg	Magnesium
MgSO <sub>4</sub>	Magnesium sulfate
Mn	Manganese
MT	Metallothionein
NaAsO <sub>2</sub>	Sodium <i>meta</i> -arsenite
Ni	Nickel
NiCl <sub>2</sub>	Nickel chloride
NGM	Nematode growth medium
PC	Phytochelatin
PCS	Phytochelatin synthase
Real-time PCR	Real-time polymerase chain reaction
ROG	Ras activating factor in development of germline gene
ROS	Reactive oxygen species
SOD	Superoxide dismutase
ZIP	Zrt-, irt-like protein
Zn	Zinc
ZnT/CDF	Cation diffusion facilitator
ZnCl <sub>2</sub>	Zinc chloride



## Acknowledgements

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It is January 17, 2014. For most people this day will not be different from yesterday or tomorrow, but for me it is due date. More particularly, it is the deadline of my Master's thesis! The icing on the cake to earn my master's degree in Biomedical Sciences. This thesis is an embracement of the laboratory work I did in the Klaunig lab at Indiana University (Bloomington, IN, USA). This also gives me the opportunity to express some words of gratitude to several persons who helped me to get this far.

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## Abstract

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**Introduction** The potential protective effect of zinc on the toxicity of arsenic, cadmium, copper, and nickel on the reproductive ability of *Caenorhabditis elegans* was investigated. Heavy metals have been addressed as toxic to different organ systems, including the reproductive system. During their evolution, organisms have developed several detoxification systems such as glutathione, metallothioneins, heat shock proteins, and transporters to defend themselves. Zinc is a trace element with well-known antioxidant properties that is tightly controlled. It is hypothesized that zinc reduces metal-induced oxidative stress, resulting in beneficial effects on the reproductive system.

**Materials and Methods** The effects of these metals on mortality, reproduction, and stress- and reproduction-related gene expression of *C. elegans* were investigated under laboratory conditions. The metal sensitivity was obtained through median lethal concentration testing, in which the mortality rate was assessed after a long term exposure (one to three days). The effect of zinc coadministration was tested on worms that were exposed for 24 hours. The metal sensitivity was also derived through examination of the egg-laying capability of the nematodes after exposure to sublethal metal concentrations. Once again, zinc was added to determine its influence on the reproduction ability of *C. elegans*. At the molecular level both the role of stress-related genes (*ape*, *ctl*, *cyp*, *gst*, *hsp*, *mtl*, *skn*, and *sod*) as well as reproduction related genes (*egl*, *gld*, *gon*, and *rog*) were examined using real-time PCR.

**Results** The 24 hours median lethal concentrations of arsenic, cadmium, copper, and nickel in *C. elegans* were 5.32, 6.26, 3.56, and 8.18 mM, respectively. With zinc coadministration these concentrations were respectively 6.62, 7.06, 6.26, and 10.17 mM. Alterations in brood sizes were observed in cadmium and copper exposed worms. Each metal treatment was related to a 100% hatching probability after 48 hours. Zinc coadministration with copper resulted in decreased brood sizes compared to only copper-treated worms. Cadmium exposure led to an increase in the expression of most of the stress-related genes tested, except for superoxide dismutase 2, skinhead 1 and apoptosis enhancer 1 genes where no significant changes were observed. Cytochrome p450 family protein 35A2 showed a decreased expression after cadmium exposure. The responses of the stress-related genes to arsenic, copper, and nickel exposure were not as intense as their responses to cadmium exposure. Zn coadministration had varying effects on the gene expression profiles. A decrease of oxidative stress was seen for cadmium and nickel exposure cotreated with zinc. In contrast, an increase was seen for copper and arsenic.

**Discussion** This study demonstrated the protective ability of zinc on metal-induced mortality. It was found that the nematodes were more sensitive when subjected to increased doses and/or when the duration of exposure to the toxicant was prolonged. A defect of the egg-laying muscles, the egg production or fertilization is suggested to cause decreased brood sizes. This study did not assess the protective effects of zinc on reproduction. The stress responses for cadmium and copper were inversely related to the observed brood sizes. Zinc cotreatment had different outcomes on the gene expression profiles. However, the protective effects of zinc on reproduction by reducing oxidative stress could not be established.



## Samenvatting

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**Introductie** De potentieel beschermende effecten van zink op de toxiciteit van arseen, cadmium, koper en nikkel op het voortplantingsvermogen van *Caenorhabditis elegans* werden onderzocht. Zware metalen zijn schadelijk voor verschillende orgaansystemen, zoals het voortplantingsstelsel. Tijdens hun evolutie hebben organismen verschillende detoxificatie systemen zoals glutathion, metallothioninen, *heat shock* proteïnen en transporters ontwikkeld om zichzelf te beschermen. Zink is een sporelement met welgekende anti-oxidatieve eigenschappen dat streng gecontroleerd wordt. Er wordt verondersteld dat zink de metaal-geïnduceerde oxidatieve stress vermindert, wat resulteert in positieve effecten op het voortplantingsstelsel.

**Materiaal en Methoden** De effecten van deze metalen op sterfte, voortplanting en stress- en voortplanting-gerelateerde genexpressie van *C. elegans* werden onder laboratorische condities onderzocht. De metaalgevoeligheid werd verkregen via *median lethal concentration* testen, waarin het sterftcijfer na een lange termijn blootstelling (een tot drie dagen) werd bepaald. Het effect van gezamenlijke zink toediening werd op wormen, die gedurende 24 uur werden blootgesteld, getest. De metaalgevoeligheid werd ook bekomen door onderzoek van het eierlegend vermogen van de nematoden na blootstelling aan subletale metaalconcentraties. Opnieuw werd zink toegevoegd om de invloed op het voortplantingsstelsel van *C. elegans* te bepalen. Op moleculair niveau werden zowel stress-gerelateerde genen (*ape, ctl, cyp, gst, hsp, mtl, skn, and sod*) als voortplanting-gerelateerde genen (*egl, gld, gon, and rog*) onderzocht door *real-time PCR*.

**Resultaten** De 24 uur *median lethal concentration* van arseen, cadmium, koper en nikkel in *C. elegans* waren respectievelijk 5.32, 6.26, 3.56 en 8.18 mM. Met gezamenlijke zink toediening waren de concentraties respectievelijk 6.62, 7.06, 6.26 en 10.17 mM. De veranderingen in *brood size* werden onderzocht in cadmium en koper blootgestelde wormen. Elke metaaltoediening was gerelateerd met 100% *hatching probability* na 48 uur. Gezamenlijke zink toediening met koper resulteerde in een verlaagde *brood size* in vergelijking met koper blootgestelde wormen. Cadmium blootstelling leidde tot een verhoogde genexpressie van de meeste stress-gerelateerde genen, behalve voor de superoxide dismutase 2, *skinhead 1* en *apoptosis enhancer 1* genen waarvoor geen significante verschillen gevonden werden. Cytochroom p450 proteïne familie 35A2 vertoonde een verlaagde genexpressie na cadmium blootstelling. De reacties van de stress-gerelateerde genen op arseen, koper en nikkel waren niet zo intens als hun reacties op cadmium blootstelling. Gezamenlijke zink toediening had afwisselende effecten op de genexpressie profielen. Een verlaging van oxidatieve stress werd vastgesteld bij cadmium en nikkel toediening samen met zink. In tegenstelling werd er een verhoging vastgesteld bij koper en arseen.

**Discussie** Deze studie toonde de beschermende eigenschappen van zink op metaal-geïnduceerde sterfte aan. Nematoden waren gevoeliger wanneer ze blootgesteld werden aan verhoogde dosissen en/of wanneer de blootstellingstijd aan de toxische stof verlengd werd. Een defect van de eierleggende spieren, de eiproductie of de vruchtbaarheid zijn waarschijnlijk oorzaken van de verlaagde *brood size*. Deze studie kon de beschermende effecten van zink niet vaststellen. De stress reacties van cadmium en koper zijn omgekeerd evenredig aan de geobserveerde *brood size*. Gezamenlijke zink toediening heeft verschillende uitkomsten op de genexpressie profielen. Echter, de beschermende effecten van zink op de voortplanting door het verminderen van de oxidatieve stress konden niet worden bevestigd.



# Introduction

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## ***Metal toxicity***

Metals, originating from their natural occurrence in the earth's crust, as well as from anthropogenic sources, create the potential for significant human exposure and therefore are of great toxicological concern. They have a tendency to accumulate in specific tissues in the human body, and even at low levels of exposure they can exert adverse effects on human health<sup>[1]</sup>. In the list of all hazards, a ranking compiled by U.S. Agency for Toxic Substances and Disease Registry (ATSDR) in 2011, the metals arsenic, lead, mercury, and cadmium take up the first, second, third and seventh place respectively, indicating the importance of elucidating the mechanisms of metal toxicity<sup>[2]</sup>. Toxic effects of various metals have already been studied extensively in both epidemiological and animal research<sup>[1, 3-7]</sup>. A higher prevalence in human cancer incidence has been found after exposure to arsenic (As), beryllium, cadmium (Cd), chromium, lead, nickel (Ni), and several other metals<sup>[8-13]</sup>. Respiratory and lung cancers are frequently occurring due to the high abundance of metals in the air by e.g. mining, smelting, alloy processing, fuel combustion, and waste incineration. Metals ingested through food and drink can also be harmful. It has been repeatedly reported that there is a relation between the heavily arsenic-contaminated deep-water wells in Taiwan and Chile, the shallow wells in Bangladesh and West Bengal (India), and cancer<sup>[14-16]</sup>.

## Cellular uptake and detoxification systems

Metals can exert either detrimental or beneficial effects in the cell, but first they must enter the cell. The plasma membrane can be passed by metal ions alone or by metals in complex with other proteins (molecular mimicry). Organisms have developed several transport mechanisms to get nutrient metals, such as cobalt, copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn), into the cell to maintain their intracellular concentrations at optimal levels needed for growth and metabolism. Zrt-, irt-like protein (ZIP) and cation diffusion facilitator (ZnT/CDF) are two major transporter families responsible for cytoplasmic Zn<sup>2+</sup> uptake and release. Zn can also enter through copper transporter 1 (CTR1) and divalent metal transporter 1 (DMT1). As the name infers, CTR1 is the primary uptake mechanism of Cu and is also capable of absorbing Fe. Like Zn, Fe and Mn can be transported through DMT1. Nonessential metals often enter the cells through competition with these transporters. Cd enters the cell through competing with Zn for ZIP, but is also absorbed via DMT1 and amino acid/cation/anion transporters. Arsenic on the other hand uses transporters whose primary function is not the transport of metals, such as aquaglyceroporins and glucose transporter 1 and 2<sup>[17, 18]</sup>. Ni does not possess specific uptake mechanisms and therefore Ni<sup>2+</sup> ions probably enter the cells by way of Ca<sup>2+</sup>/Mg<sup>2+</sup> channels<sup>[19]</sup>.

Organisms have also developed cellular detoxification systems to regulate their intracellular metal concentrations. Glutathione (GSH) possesses antioxidant properties and functions by reducing metabolites and enzyme systems<sup>[20]</sup>. GSHs have been reported to increase, decrease, or remain constant after exposure to metals<sup>[21-23]</sup>. Additionally, in the presence of metal ions phytochelatin (PCs) are synthesized enzymatically from GSH by phytochelatin synthase (PCS). Metallothioneins (MTs) are a different kind of metal binding proteins, but their occurrence is regulated at the transcriptional level. MTs bind and sequester both metals of physiological importance, as well as nonessential metals. This MT-metal complex may be transient, because MTs can release the metal ions rapidly. Heat shock proteins (HSPs) are ATP-binding proteins that can bind metals, resulting in inactivation and prevention of protein aggregation. Another non-protein mechanism to detoxify the cells is the existence of metal pumps and transporters<sup>[20]</sup>. Multidrug resistance-associated protein, two members of the P-glycoprotein subfamily (PGP1 and PGP3), and half molecule transporter are examples of ATP-binding cassette transporters that contribute to the detoxification of heavy metals<sup>[20, 24]</sup>.

### Physiological effects of metals

It has been well documented that metals affect a variety of organ systems. Metals may either suppress or stimulate the immune cell activity, causing several manifestations such as hypersensitivity, chronic inflammation, cancer development, and allergic and autoimmune diseases<sup>[25]</sup>. Hypertension and cardiovascular and cerebrovascular disease (CCVD) are just two examples of the numerous adverse effects of metals on the cardiovascular system<sup>[26, 27]</sup>. As mentioned above, the metals can enter the body through ingestion, what makes the gastrointestinal tract a direct target. Nausea or vomiting, abdominal pain, and diarrhea are a few symptoms that can occur upon metal exposure in humans<sup>[28]</sup>. The nervous system is yet another system that can be under attack. Dysfunction of the nervous system is a result of the gradual and progressive loss of neuronal cells<sup>[29]</sup>. Many studies have shown evidence of the involvement of metals in several neurologic diseases, including Parkinson's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, autism, and stroke<sup>[29-35]</sup>. There exist several theories about what underlies these diseases: dysfunction of the mitochondria, dysfunction of the glutamate transport system, defective antioxidative enzymes, etc. But there is one thing they all have in common, an increase in oxidative stress followed by the removal of neurons through apoptotic or necrotic processes<sup>[36, 37]</sup>. On the reproductive system of the human being metals can cause adverse effects either directly, by targeting certain reproductive organs, or indirectly, when acting on the neuroendocrine system<sup>[38]</sup>. Reproductive toxicity encompasses both defects on sexual function and fertility in adult organisms, as well as developmental toxicity in their progeny<sup>[39]</sup>. Stassen *et al.* (2012) explored the relationship between reproductive disorders and metal exposure in the Weenhayek, a tribe who lives along the heavily polluted Pilcomayo River (Bolivia) caused by upstream mining activity. They found indications for increased risk of smaller families, due to impaired male fertility<sup>[40]</sup>. The awareness regarding these adverse reproductive health effects due to chemical exposure has been growing throughout the last few decades. Recently, some trends in fertility and reproduction have been found; more specifically, (1) an increase in testicular cancer incidents, (2) decreasing sperm counts, (3) a decline in serum testosterone, (4) earlier pubertal development in girls, (5) fewer males being born, and (6) a documented increase in certain types of birth defects were associated with increased metal exposure<sup>[41]</sup>.

The reproductive system was chosen in this study as research subject, because of the adverse effects of metals on both the parents and their progeny.

### Cellular and molecular effects of metals on reproduction

Metals can exert adverse reproductive effects through several mechanisms, including damaging of the oocytes and sperm cells, interference with cell functions (e.g. protein folding and stress signaling pathways), or changing of the DNA structure<sup>[41, 42]</sup>. If the oocytes or sperm cells are targeted infertility can arise and when other type of cells are killed or damaged developmental problems can occur. An altered structure of DNA causes gene mutations, resulting in an inability to conceive or in birth defects in the offspring<sup>[41]</sup>. Oxidative stress, a disturbance in the balance between pro-oxidants and antioxidants, has been identified as a key player in the pathogenesis of subfertility in both males and females. In men oxidative stress is responsible for impaired sperm quality and function. The impact of oxidative stress on the reproductive ability of women is rather unclear, although animal and *in vitro* models show that oxidative stress is associated with a decrease in female fertility<sup>[43, 44]</sup>. Environmental chemicals including pesticides, phthalates, and metals are known to trigger oxidative stress through excess production of reactive oxygen species (ROS), resulting in e.g. DNA damage, lipid peroxidation, depletion of sulfur-containing proteins (e.g. GSH, and *N*-acetylcysteine), and inhibition of DNA repair<sup>[45-47]</sup>. The primary mechanism of toxicity for Cd, Ni, and mercury is depletion of glutathione and their high affinity for sulfhydryl (-SH) groups. Arsenic on the other hand is thought to exert its toxic effects not only by binding directly to -SH groups, but also through formation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)<sup>[47]</sup>. All organisms feature an antioxidant defense system which is activated through changes in H<sub>2</sub>O<sub>2</sub> or superoxide (O<sub>2</sub><sup>•-</sup>) levels<sup>[48]</sup>. Antioxidants can dispose, scavenge, or suppress the formation of ROS. Superoxide dismutase (SOD), catalase

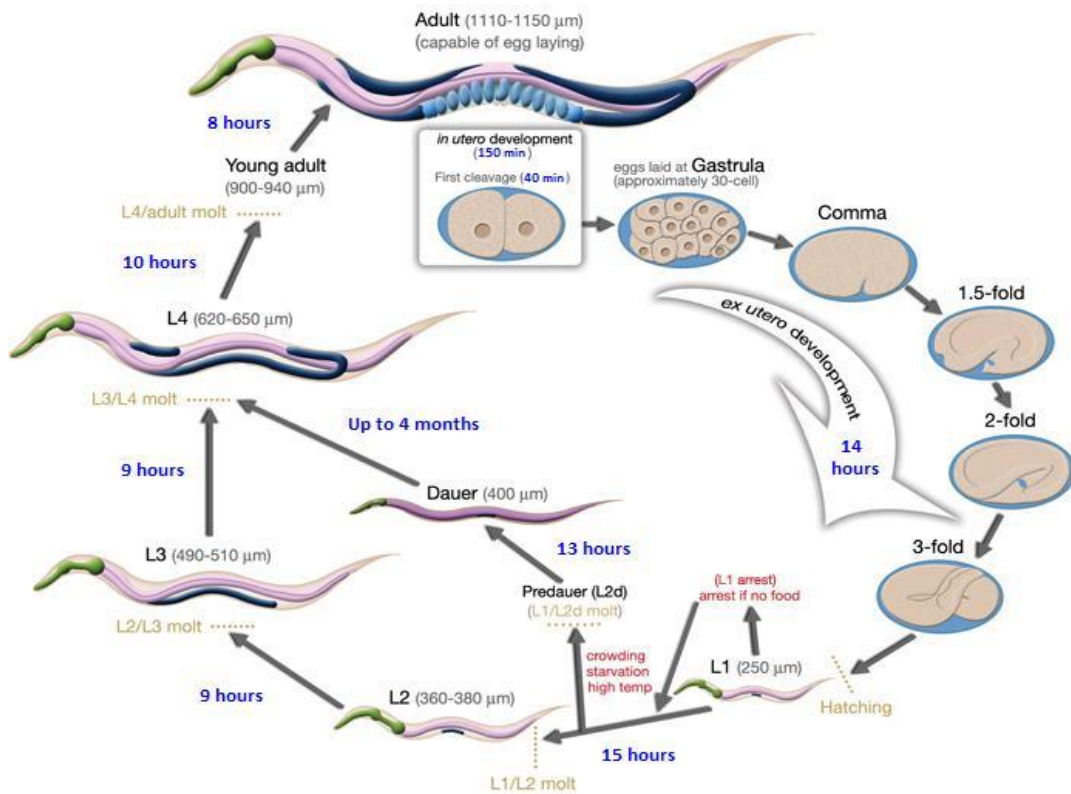


Fig. 1 Life cycle of *C. elegans* at 20°C. (Adapted from <http://www.wormatlas.org>)

(CAT), glutathione peroxidase, and glutathione oxidase are examples of enzymatic antioxidants, while GSH, vitamins C and E, and carotenoids represent the class of non-enzymatic antioxidants<sup>[43, 44, 49]</sup>.

### ***Zinc: a crucial player in the oxidative stress response***

An element that is also well-known for its antioxidant properties is Zn. It is an essential trace element that participates in metabolic and signaling pathways as a cofactor for enzymatic reactions. To secure the supply of Zn for structural and catalytic functions cells need to absorb Zn actively. However, elevated cellular concentrations of Zn are toxic. Therefore excess Zn is transported out of the cell and stored into vesicles or bound to MTs<sup>[47]</sup>. Several studies on the protective effects of Zn against chemical-induced oxidative stress in vertebrates are available, but so far these findings have not been addressed in invertebrates<sup>[50-53]</sup>.

### ***The nematode *Caenorhabditis elegans* as model organism***

To study the effects of metals on the human body, several model organisms have been introduced over the years, including rodents and fish. More recently, *Caenorhabditis elegans* has been accepted as a good model system. The establishment of *C. elegans* as model organism began in 1963 with the efforts of Sydney Brenner<sup>[54]</sup>. *C. elegans* is a small, free-living nematode (roundworm) mainly residing in the interstitial water of temperate soil environments all over the world<sup>[54, 55]</sup>. *C. elegans* survives by feeding primarily on bacteria, but is also found in various microbe-rich habitats such as decaying plant matter<sup>[56]</sup>. Its many advantages for research include small size (~1.5 mm adult), rapid life cycle (~3 days), short lifespan (~3 weeks), self-fertilization, and well-defined genetic, physiological, molecular, and developmental stages (Fig. 1)<sup>[54, 57]</sup>. Besides its simplicity, many of its fundamental cellular and molecular structures and biological characteristics are similar with higher



organisms, such as humans. Approximately 60%-80% of human genes have homologues in *C. elegans*<sup>[20, 57, 58]</sup>. Even more so, *C. elegans* possesses the same metal detoxification systems as humans, and therefore is a very attractive organism to study metal toxicology. Previous toxicity studies also revealed the high predictive value of *C. elegans* for mammalian systems<sup>[57, 59, 60]</sup>. The metal toxicity tests commonly performed in *C. elegans* are mortality (e.g. lethality and lifespan), behavior (e.g. head thrashes, body bends, and chemotaxis), reproduction (e.g. brood size and hatch time), development (e.g. body length and neuron number), and molecular changes (e.g. DNA damage and repair and gene expression).

Intensive studies have been conducted to elucidate the role of metals on the reproductive ability of *C. elegans*. The parameters associated with reproduction are hatch time (defined as the day during which an embryo hatched into a larva), hatching probability (defined as the number of larvae present at hour 24 to 26 divided by the number of progeny present at the end of exposure) and brood size (defined as the number of progeny of the parental worm). Hatch time is frequently used as a parameter for reproductive ability in several organisms, such as chick, carp, and lizard embryos<sup>[61-63]</sup>. Lin *et al.* (2013) are the only ones who reported hatch time as a parameter to examine the reproductive ability in *C. elegans* after ethanol exposure<sup>[64]</sup>. They, together with Davis *et al.* (2008), did also explore hatching probability in *C. elegans* after ethanol exposure<sup>[64, 65]</sup>. However, hatch time and hatching probability have never been studied for metals in *C. elegans*. Furthermore, it is known that metals reduce brood size in *C. elegans* in a concentration-dependent manner<sup>[66-68]</sup>. As mentioned earlier, oxidative stress is related to the changes in reproductive ability, and therefore a great deal of research has been focused on the expression and regulation of stress-related genes to explore the role of environmental exposure in affected reproductive ability. HSP, MT, vitellogenin, cytochrome P450 family protein 35A2, glutathione-S-transferase (GST), SOD, and CAT are some of the gene classes that are related to the stress response in *C. elegans*, and have been studied innumerable times. In contrast genes directly related to the reproductive ability, such as egg-laying defective (EGL), abnormal gonad development (GON), Ras activating factor in development of germline (ROG), and defective in germ line development (GLD), were never studied for metal exposure. This study is an opportunity to discover the role of these genes on the metal-induced adverse effects on the reproductive system.

### **Aim of the study**

In this study, the effect of Zn on the reproductive ability of *C. elegans* after exposure to several metals was investigated. The hypothesis of this project is that Zn reduces oxidative stress after metal exposure in *C. elegans*, and accordingly reduces the adverse effects of metals on the reproductive system.

The first objective of this study was to determine the effect of metals on the survival rate, following concomitant administration of Zn. Four metal compounds were selected for this study: As, Cd, Cu, and Ni. First, wild type *C. elegans* were exposed to a concentration range (0.05, 0.5, 1, 5, and 10 mM) of different metals. After 24 hours the worms were scored as dead or alive. In order to reach the objective, Zn was added to the metal lethal concentrations in order to explore the potential of Zn to rescue *C. elegans* from death. In a preliminary study, different concentrations of Zn were tested for its toxicity. 0.1 mM Zn was shown as a concentration that did not affect the survival rate nor the brood size of the worms.

Secondly, the effect of Zn addition on the reproductive ability was investigated in this project. Wild type *C. elegans* worms were exposed to sublethal metal concentrations (0, 0.05, 0.1 mM Cd/Ni; 0, 0.1, 0.5 mM Cu; 0, 0.1, 1 mM As), which were determined during the first objective, whereupon hatching probability and brood size were determined. Afterwards, if a significant decrease in brood size was noticed after exposure to a certain metal, Zn was coadministered and once again hatching probability and brood size were determined.

The last objective of this project was to study the effects of Zn coadministration on the expression of several genes involved in oxidative stress regulation and reproduction in *C. elegans*. Again, wild type *C. elegans* were exposed to sublethal metal concentrations (0.1 mM Cd/Ni; 0.5 mM Cu; 1 mM As) for 12 hours, respectively

with and without the addition of Zn. Subsequently, the expression of the genes of interest (*hsp70*, *hsp16.1*, *hsp16.2*, *hsp16.48*, *mtl1*, *mtl2*, *ctl1*, *ctl2*, *sod2*, *sod3*, *sod4*, *skn1*, *ape1*, *gst4*, *gst38*, *cyp35A2*, *elg1*, *elg10*, *gld1*, *gld2*, *gon4*, *gon14*, and *rog1*) was determined using real-time polymerase chain reaction (real-time PCR).



# Materials and methods

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## ***C. elegans maintenance***

The wild type *C. elegans* Bristol strain N2 was used in the present study, and was originally obtained from the *Caenorhabditis* Genetics Center (Twin Cities, MN). The worms were maintained at 20°C on nematode growth medium (NGM) or 8P agar plates seeded with *Escherichia coli* strains OP50 and HB101, respectively, according to standard methods<sup>[55, 69]</sup>. In all experiments 60 mm polystyrene petri dishes (Sigma-Aldrich) were used and filled with 10 ml medium. NGM is a mixture of 3 g NaCl, 17 g bacto agar, 2.5 g bacto peptone, 12.5 mM KPO<sub>4</sub> buffer pH 6.0 (108.3 g KH<sub>2</sub>PO<sub>4</sub>, 35.6 g K<sub>2</sub>HPO<sub>4</sub>, H<sub>2</sub>O to 1 l), 1 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1 ml cholesterol (5 mg/ml in ethanol), and 1 l H<sub>2</sub>O. 8P medium varies from NGM through the addition of 25 g bacto agar and 20 g bacto peptone<sup>[70]</sup>.

## ***Chemicals***

All chemicals, except Cd, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cadmium chloride was purchased from Fisher Scientific (Fair Lawn, NJ, USA).

## ***Metal exposure and sample preparation***

Three types of endpoints (mortality, reproduction, and stress- and reproduction- related gene expression) were assessed for exposure to As, Cd, Cu, and Ni, with and without the addition of Zn. The nematodes were exposed to sodium arsenite (NaAsO<sub>2</sub>), cadmium chloride (CdCl<sub>2</sub>), copper sulfate (CuSO<sub>4</sub>), nickel chloride (NiCl<sub>2</sub>·6H<sub>2</sub>O), and zinc chloride (ZnCl<sub>2</sub>) through the NGM. Young adults (age, 3 days) from an age-synchronized culture were used in all experiments including both control and treatment groups. To obtain age-synchronized worms, gravid adults were treated with an alkaline bleach solution freshly made before experiments. The embryos were incubated in M9 buffer for 18 hours, and then washed 3 times in deionized water using standard protocols<sup>[70, 71]</sup>.

## ***Lethality tests***

Six different metal concentrations (0, 0.05, 0.5, 1, 5, and 10 mM) at three time points (24, 48, and 72 hours) were used for the lethality test. Three concentrations (0, 5, and 10 mM) were used for testing the protective effects of 0.1 mM Zn. Using a dissecting microscope (Leica, M165 FC, USA), animals were counted and scored as dead or alive at the specified time points. Viability was scored based on the ability to move in response to poking with a platinum wire. Worms that could only move their head, were also recorded as dead. The LC10, LC50, and LC90 values, the concentrations for which respectively 10%, 50%, or 90% of the worms died, were calculated using probit-analysis (SAS9.4). The experiments were replicated two to four times, and fifteen worms were used for every condition.

## ***Hatching probability and brood size analysis***

Three different sublethal concentrations for each metal were selected for testing reproduction deficiencies (0, 0.05, 0.1 mM Cd/Ni; 0, 0.1, 0.5 mM Cu; 0, 0.1, 1 mM As). In a next experiment, 0.1 mM Zn was added to explore the protective ability of Zn on reproduction. One young adult was placed on each experimental plate, and transferred to a fresh plate after 24 hours to ensure exposure to a consistent metal concentration throughout development. After 48 hours the worms were removed from the experiment. Plates of eggs collected after exposure were observed every 24 to 26 hours under a dissecting microscope (Leica, M165 FC, USA). Larvae found at the time of observation were recorded as hatched during that day and removed from the

**Table 1 Real-time PCR primers.** From top to bottom: the reference gene *actin*, the oxidative stress-related genes, and the reproduction-related genes. Each gene is listed with its abbreviation, accession number, forward and reverse primers presented in the 5' to 3' direction, and their amplicon length (bp).

Gene class	Abbreviation	Accession No.	Forward primer	Reverse primer	Amplicon length (pb)
Actin	<i>act1</i>	P0DM11	ACCATGTACCCAGGAATTGC	TGGAAGGTGGAGAGGGAAG	110
Heat shock protein	<i>hsp70</i>	O45246	GCTGATCTTTCCGCAAGAC	GTTGAGGTCCCTCCATTGA	157
Heat shock protein	<i>hsp16.1</i>	P34696	CCACTATTTCCGTCCAGCTC	GGCTTGAAGTGCAGACATT	131
Heat shock protein	<i>hsp16.2</i>	P06582	CTCAACGTTCCGTTTTTGGT	CGTTGAGATTGATGGCAA	98
Heat shock protein	<i>hsp16.48</i>	P02513	CATGCTCCGTTCTCCATTTT	TGGTTTGAATGAGAAACATCG	148
Metallothionein	<i>mtl1</i>	P17511	TGGATGTAAAGGGAGACTGCAA	CATTTTAATGAGCCGCAGCA	66
Metallothionein	<i>mtl2</i>	P17512	AAGTGTGCCAACTGCGAATGT	GCTTTCAAGAAAAACCTCGA	70
Catalase	<i>ctl1</i>	O61235	CTCCTACACGGACACGCAT	GCATCTCCCTGGCTTTCAT	94
Catalase	<i>ctl2</i>	Q27487	CGAACAGCTTCAACTATGG	GTGGCTGGGAATGTGGTAT	23
Superoxide dismutase iron/manganese	<i>sod2</i>	P31161	GGCATCAACTGTCGCTGT	ACAAGTCCAGTTGTTGCC	87
Superoxide dismutase iron/manganese	<i>sod3</i>	P41977	TGACATCACTATTGCGGT	GGGACCATTCTTCCAAA	81
Superoxide dismutase copper/zinc	<i>sod4</i>	P34461	CACCAGATGACTCGAACA	AATGAGGCAAGAGAGTCG	64
Skinhead	<i>skn1</i>	P34707	GACGTCAATTTATGGAGTGTCC	GAAGATGTTTTGTCGTGATCCG	93
Apoptosis enhancer	<i>ape1</i>	Q9XVN3	CACATCCACAAGCATTGTCC	ATCGGCGATCATTTCAGAAG	192
Glutathion S-transferase	<i>gst4</i>	Q21355	TGCTCAATGTGCCTTACGAG	AGTTTTTCCAGCGAGTCCAA	133
Glutathion S-transferase	<i>gst38</i>	O45451	TGATTTGCTGGGACGTGAAA	CTGGACGAGTCTCAATCCATT	75
Cytochrome P450 family	<i>cyp35A2</i>	O02628	GGTGGCATTGTTTCGACTCT	TGTCCGCATATTTACCAGC	170
Egg-laying defective	<i>egl1</i>	O61667	CAGGACTTCTCCTCGTGTGAAGATTC	GAAGTCATCGCACATTGCTGCTA	77
Egg-laying defective	<i>egl10</i>	P49809	ACCGGACAAGATCTGATTGG	AACAATTTGACGGCCAAAAG	150
Defective in germline development	<i>gld1</i>	Q17339	GACTCCCGTCAACGACATTT	GGTACTTCGGGAACGTCAAA	122
Defective in germline development	<i>gld2</i>	O17087	GGCGAGTATTCTGGGAATCA	CGTAACCCTGGGTCGAGTTA	93
Abnormal gonad development	<i>gon4</i>	Q21216	ACCACGCCTTGAAAATTACG	TTCTTTGACGCCCGATAAAC	99
Abnormal gonad development	<i>gon14</i>	H2L033	ATCCAAAGAGGGCATTGTTG	ATCTTTTCCGCGAATCACAC	74
Ras activating factor in development of germline	<i>rog1</i>	A3KBY2	ATTGAAACGGGTCTCACAC	CTTCCACTCTGGGATCAAA	139

plate. Hatching probability was defined as the number of larvae present at hour 24 to 26 divided by the number of progeny present at the end of exposure. Brood size was measured as the total number of eggs laid<sup>[72]</sup>. The experiments were replicated four times.

### **Gene expression**

Young adults were exposed for 12 hours to the highest metal concentration used in the brood size analysis (0.1 mM Cd/Ni; 0.5 mM Cu; 1 mM As). Total RNA was extracted from a synchronized *C. elegans* population using Trizol reagent as described previously, with some slight modifications<sup>[73]</sup>. Specifically, worms were collected from treatment or control plates by washing with water. The worm pellets were resuspended in Trizol (Life Technologies) (1 ml/100  $\mu$ l compact worm pellet), snap frozen in liquid nitrogen and stored at -80°C until all the samples were collected. Chloroform (Sigma-Aldrich) was used to separate RNA from protein and other materials, and total RNA was precipitated with isopropyl alcohol (Fisher Scientific). The RNA pellet was washed once with 70% ethanol and air-dried. The resultant RNA was dissolved in RNase-free water (Hoefer), and stored at -80°C for further analysis. The RNA concentrations were measured using a Nanodrop ND-2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE), and all samples were adjusted to 2  $\mu$ g. Genomic DNA elimination and reverse transcription were performed with the QuantiTect Reverse Transcription Kit (Qiagen) following manufacturer's protocol. Briefly, gDNA wipeout buffer (7x) was added to 2  $\mu$ g RNA and further diluted with RNase free water to a total reaction volume of 28  $\mu$ l. After a 2 minutes incubation period at 42°C, reverse transcription was performed. Reverse transcriptase, RT buffer (5x), and RT primer mix were added to make a total volume of 40  $\mu$ l, and the mixture was incubated for 15 minutes at 42°C prior to incubation for 3 minutes at 95°C to inactivate reverse transcriptase (Bio-Rad DNA Engine Peltier Thermal Cycler). The cDNA was diluted 10 times before storage at -20°C.

The primer sequences of the genes were found in literature or designed using the *C. elegans* genome database (Table 1)<sup>[74]</sup>. The expression of several genes involved in oxidative stress or reproduction in *C. elegans* was examined. Real-time PCR was performed using a 7900HT Fast Real-Time PCR System with 96-well plate (Applied Biosystems, Bedford, MA) under universal thermal cycling conditions (20 seconds at 95 °C, 45 cycles of 3 seconds at 95°C and 30 seconds at 60°C). Each 10  $\mu$ l reaction contained Fast SYBR Green Master Mix (2x) (Applied Biosystems), 1  $\mu$ M of a gene-specific forward and reverse primer, and 1.5  $\mu$ l of the 1/10 diluted cDNA. Gene expression was normalized to *actin* and data was calculated using the  $2^{-\Delta\Delta Ct}$  method. A total of six replicates for each condition was performed: 3 biological replicates with each a technical duplicate.

### **Data analysis**

For lethality and brood size experiments, all statistical significances of differences between means compared to control were determined using the parametric Student's *t*-test. The statistical power for each non-significant brood size effect was estimated post-hoc by means of GPower 3.0.10 software (Erdfelder, Faul, Lang and Buchner, 2008). For gene expression changes, the statistical significance of differences between means was determined by means of a one-way analysis of variance (ANOVA), followed by Tukey-Kramer post-hoc test. Finally, a nonparametric Kruskal-Wallis test was performed when the data were not normally distributed. *P*-values less than 0.05 were considered statistically significant. Statistics were performed using GraphPad Prism 6.02 (GraphPad Software Inc., 2013) and SAS 9.4 software (SAS Institute Inc., 2013).



## Results

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The purpose of this study was to investigate the effect of Zn on the reproductive ability of the heavy metal-exposed nematode *C. elegans*. Both the overall metal toxicity at the level of the organism as well as the underlying molecular mechanisms were studied.

The metal sensitivity was obtained through LC50 testing, in which the mortality rate was assessed after a long term exposure (one to three days). The effect of Zn coadministration was tested on worms that were exposed for 24 hours. The metal sensitivity was also derived through examination of the egg-laying capability of the nematodes after exposure to sublethal metal concentrations. Once again, Zn was administered to determine its influence on the reproductive ability of *C. elegans*. At the molecular level both the role of stress-related genes (*ape*, *ctl*, *cyp*, *gst*, *hsp*, *mtl*, *skn*, and *sod*) as well as reproduction related genes (*egl*, *gld*, *gon*, and *rog*) were examined using real-time PCR.

### THE EFFECT OF METALS ON SURVIVAL AND REPRODUCTION

#### ***Lethality tests and establishment of the LC value***

Mortality is an important parameter to assess heavy metal toxicity. To examine for dose-dependent toxicity to the nematode *C. elegans*, 60 young adult worms were exposed to six different concentrations (0, 0.05, 0.5, 1, 5, and 10 mM) for each metal during one to three days. The corresponding lethal concentrations (LC10, LC50, and LC90) were calculated using probit-analysis (Table 2). At 24 hours the LC50 values were 6.26 mM (confidence interval: 1.15 mM – 7.55 mM) CdCl<sub>2</sub>, 5.32 mM (4.77 mM – 5.99 mM) NaAsO<sub>2</sub>, 3.56 mM (3.08 mM – 4.13 mM) CuSO<sub>4</sub>, and 8.18 mM (7.02 mM – 9.83 mM) NiCl<sub>2</sub>; for two days the LC50 values were 0.83 mM (0.67 mM – 1.02 mM) CdCl<sub>2</sub>, 4.97 mM (4.41 mM – 5.64 mM) NaAsO<sub>2</sub>, 1.02 mM (0.90 mM – 1.19 mM) CuSO<sub>4</sub>, and 3.71 mM (2.51 mM – 5.98 mM) NiCl<sub>2</sub> and for three days the LC50 values were 0.28 mM (0.20 mM – 0.36 mM) CdCl<sub>2</sub>, 3.55 mM (3.09 mM – 4.09 mM) NaAsO<sub>2</sub>, 0.93 mM (0.83 mM – 1.05 mM) CuSO<sub>4</sub>, and 1.19 mM (0.94 mM – 1.50 mM) NiCl<sub>2</sub>. The order of chronic toxicity (LC50s), expressed in atomic mass units (molar), observed for *C. elegans* after 24 hours of exposure was Cu > As > Cd > Ni. The lethality toxicity manner for examined metals was Cd > Cu > Ni > As based on two and three days assay. Taken together, these data indicate that the metal toxicity in the nematode *C. elegans* is dependent both in time and in concentration.

#### ***Stress to the reproductive system: physiological level***

The egg-laying ability of worms exposed to metals was analyzed by counting the number of eggs per worm in control versus metal-treated worms, to gain a better understanding of the reproductive effects of these toxicants. Based on the results of the preceding chronic toxicity test, two concentrations for each metal – corresponding to 17.9% and 35.7% of the LC50 of 3 days for CdCl<sub>2</sub>; 2.8% and 28.2% of the LC50 of 3 days for NaAsO<sub>2</sub>; 10.8% and 53.8% of the LC50 of 3 days for CuSO<sub>4</sub>; and 4.2% and 8.4% of the LC50 of 3 days for NiCl<sub>2</sub> – were selected for the sublethal exposure conditions. The average number of eggs per worm in the unexposed controls was in the range of 232 to 284. The number of eggs per worm was not changed by Ni or As exposure, whereas Cd and Cu exposure resulted in a remarkable reduction in the brood size. The brood size of the animals exposed to 0.1 mM Cd, 0.1 mM and 0.5 mM Cu were reduced by 61%, 34% and 36%, respectively, compared to the control ( $p < 0.01$ ) (Fig. 2B). However, the decrease in fertility did not affect hatching probability. All progeny hatched within 48 hours after been laid (Fig. 2A). Only for Cu there was a small difference compared to the control after 24 hours (0.1 mM Cu  $p < 0.01$ , 0.5 mM  $p < 0.05$ ). These data indicate



**Table 2 The effect of metals on survival rate in *C. elegans*.** Estimation (mM) of 10% lethal concentration (LC10), 50% lethal concentration (LC50), and 90% lethal concentration (LC90) with their 95% confidence interval (CI) after 24h, 48h, and 72h of exposure to As, Cd, Cu, and Ni. The toxicity increased when the exposure time was prolonged.

	24h				48h				72h			
	Cd	As	Cu	Ni	Cd	As	Cu	Ni	Cd	As	Cu	Ni
LC10	0,96	2,50	0,87	0,22	0,19	1,87	0,44	0,16	0,09	1,10	0,42	0,26
CI	-15,39-4,12	1,72-3,10	0,25-1,37	-1,55-1,43	0,12-0,27	1,10-2,47	0,25-0,56	0,05-0,31	0,04-0,13	0,51-1,59	0,25-0,54	0,16-0,37
LC50	6,26	5,32	3,56	8,18	0,83	4,97	1,02	3,71	0,28	3,55	0,93	1,19
CI	1,15-7,55	4,77-5,99	3,08-4,13	7,02-9,83	0,67-1,02	4,41-5,64	0,90-1,19	2,51-5,98	0,20-0,36	3,09-4,09	0,83-1,05	0,94-1,50
LC90	11,56	8,15	6,25	16,14	3,59	8,08	1,60	86,65	0,91	5,99	1,43	5,39
CI	9,99-18,67	7,29-9,40	5,49-7,31	13,64-20,19	2,67-5,48	7,19-9,35	1,38-2,00	36,61-368,51	0,71-1,25	5,29-6,97	1,26-1,72	3,82-8,86

that the magnitude of toxicity on reproduction depends on the metal and the metal concentration that is used.

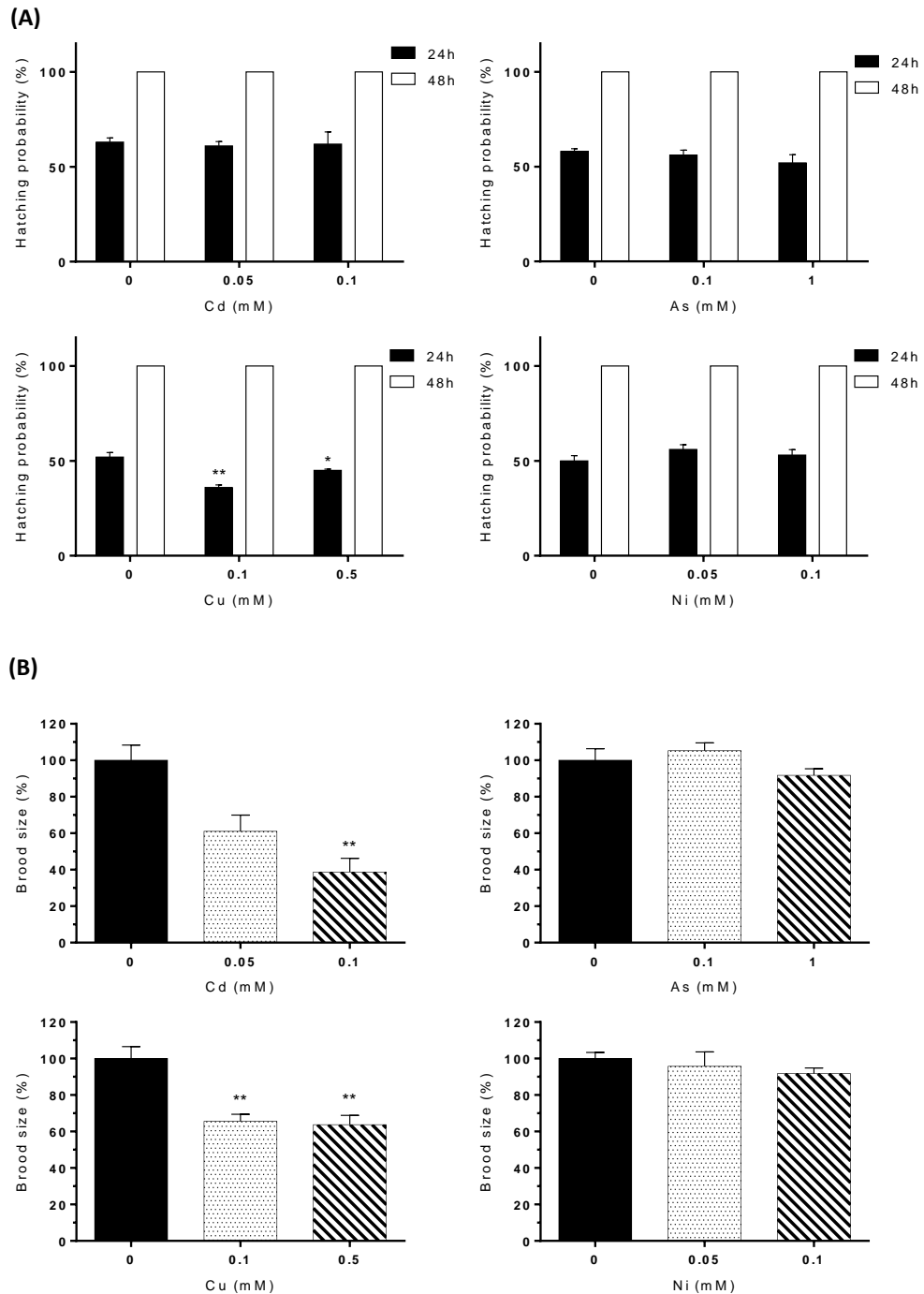
### **Stress to the reproductive system: molecular level**

The transcript levels were examined to look for changes at the molecular level of the organism. Hence, the effect of metals on gene expression patterns of heat shock protein (*hsp70*, *hsp16.1*, *hsp16.2* and *hsp16.48*), metallothionein (*mtl1* and *mtl2*), catalase (*ctl1* and *ctl2*), superoxide dismutase (*sod2*, *sod3*, *sod4*), apoptosis enhancer (*ape1*), glutathione S-transferase (*gst4* and *gst38*), cytochrome p450 family protein 35A2 (*cyp35A2*), egg-laying defective (*elg1* and *elg10*), defective in germline development (*gld1* and *gld2*), abnormal gonad development (*gon4* and *gon14*), and Ras activating factor in development of germline (*rog1*) were observed. [Figure 4](#) shows the oxidative stress-related and reproduction-related gene expression profile measured in the young adults of *C. elegans* exposed to As, Cd, Cu, and Ni for 12 hours.

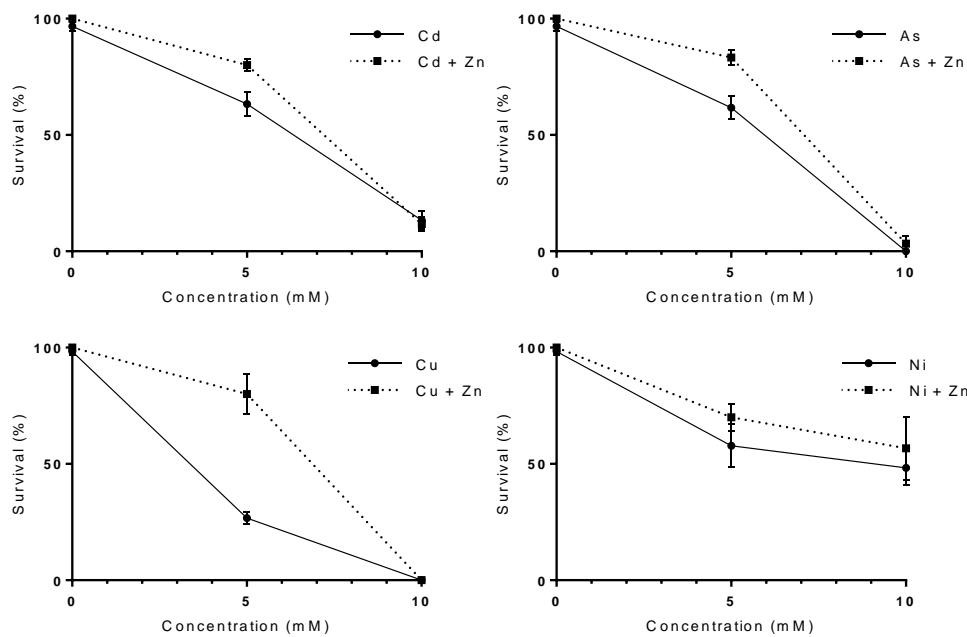
Cd exposure led to increases in the expression of all stress-related genes, except for *sod2*, *skn1* and *ape1*, where no significant changes were observed ([Fig. 4A](#)). The degree of increase was more important in *hsp70* (618.1 folds compared to the control,  $p < 0.01$ ), *hsp16.1* (666.7 folds,  $p < 0.01$ ), *hsp16.2* (351.9 folds,  $p < 0.01$ ), *hsp16.48* (308.6 folds,  $p < 0.01$ ), *mtl1* (256.6 folds,  $p < 0.01$ ), *mtl2* (15.0 folds,  $p < 0.01$ ), *ctl1* (4.4 folds,  $p < 0.01$ ), *ctl2* (4.5 folds,  $p < 0.01$ ), *sod4* (7.22 folds,  $p < 0.05$ ), *gst4* (10.6 folds,  $p < 0.01$ ), and *gst38* (529.7 folds,  $p < 0.01$ ). Cd led to a significant decrease in the expression of the *cyp35A2* gene (5.6 folds,  $p < 0.01$ ). Cd did not induce significant changes in the gene expression profile of the reproduction-related genes ([Fig. 4B](#)).

Cu exposure led to increased expression of *hsp70* (10.4 folds,  $p < 0.01$ ), *hsp16.1* (8.5 folds,  $p < 0.01$ ), *hsp16.48* (4.9 folds,  $p < 0.05$ ), *ctl1* (17.2 folds,  $p < 0.01$ ), and *sod4* (12.0 folds,  $p < 0.01$ ). Cu is responsible for a decrease in the expression of *sod3* (5.3 folds,  $p < 0.01$ ) and *gst4* (3.0 folds,  $p < 0.01$ ) ([Fig. 4A](#)). Whereas Cd exposure did not show any reproduction-related gene expression profile changes, Cu did alter the expression of the *gld2* gene (1.7 folds,  $p < 0.05$ ) ([Fig. 4B](#)).

Looking to the stress-related gene expression changes, Ni exposure resulted only in an upregulation of *ctl1* (4.6 folds,  $p < 0.01$ ) and downregulation of *gst4* (2.2 folds,  $p < 0.05$ ) ([Fig. 4A](#)). The reproduction-related genes *gld2* (2.2



**Fig. 2 The effect of metals on reproduction of *C. elegans*.** (A) Hatching probability is defined as the number of larvae present at hour 24 to 26 divided by the number of progeny present at the end of exposure. There is no significant difference in hatching probability for all metal concentrations, except for Cu within 24 h. Within 48 h all progeny hatched. (B) Brood size is defined as the number of progeny of the parental worm, and is significantly decreased in parental worms exposed to 0.1 mM Cd, 0.1 mM Cu, and 0.5 mM Cu. The brood size of the control groups (100%) of Cd, As, Cu, and Ni represents 284, 232, 239, and 271 progeny, respectively. Single synchronized young adult hermaphrodites were transferred to 60 mm Petri plates at indicated metal concentrations. Worms were transferred to a fresh Petri plate and removed from the experiment after 24 h and 48 h, respectively. Data are expressed as percent of control. All values represent means  $\pm$  SEM;  $n = 4$ . \*\* $p < 0.01$  vs. control.



**Fig. 3** The effect of Zn on survival rate of *C. elegans*. Dose-response curves of lethality after metal exposure with and without the addition of Zn. Synchronized young adult hermaphrodites were transferred to 60 mm Petri plates at indicated metal concentrations. After 24 h viability was scored based on the ability to move in response to poking with a platinum wire. Data are expressed as percent of control. All values represent means  $\pm$  SEM;  $n_{\text{without Zn}} = 60$ ,  $n_{\text{with Zn}} = 30$ .

folds,  $p < 0.01$ ) and *gon4* (2.0 folds,  $p < 0.05$ ) were overexpressed after Ni exposure (Fig. 4B).

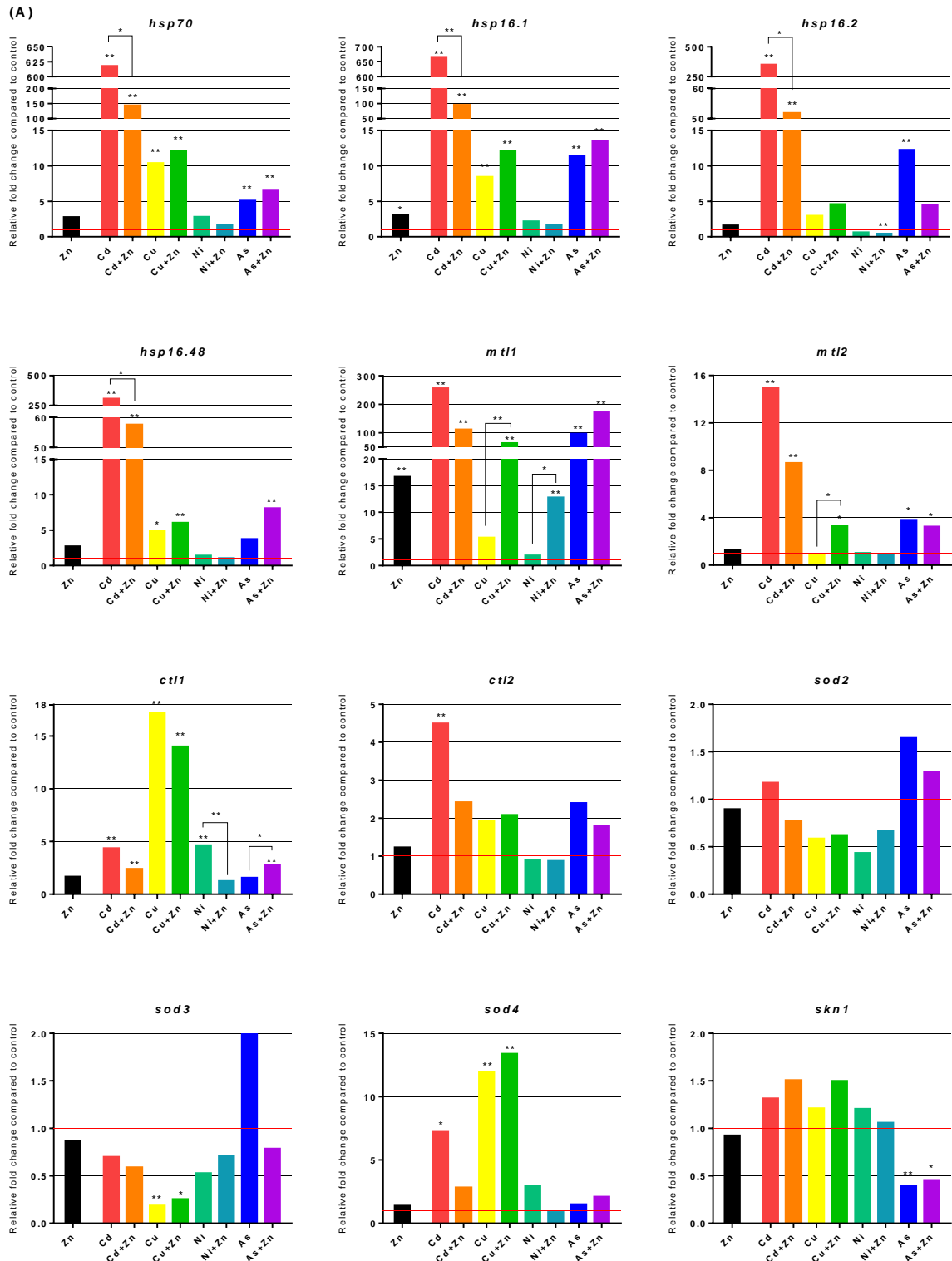
Arsenic caused an underexpression of *skn1* (2.5 folds,  $p < 0.01$ ) and induced an upregulation of several genes including *hsp70* (5.1 folds,  $p < 0.01$ ), *hsp16.1* (11.5 folds,  $p < 0.01$ ), *hsp16.2* (12.3 folds,  $p < 0.01$ ), *mtl1* (97.3 folds,  $p < 0.01$ ), *mtl2* (3.8 folds,  $p < 0.05$ ), *gst4* (10.6 folds,  $p < 0.01$ ), and *gst38* (529.7 folds,  $p < 0.01$ ), whereas no difference in the expression of *hsp16.48*, the CTL and SOD gene family classes, *ape1* and *cyp35A2* can be found (Fig. 4A). Arsenic exposure significantly upregulated the expression of the reproduction-related gene *gon4* (2.1 folds,  $p < 0.05$ ) (Fig. 4B).

These data indicate that the stress-related gene expression profile of the nematodes is affected most by the noxious metals, and that these metals almost do not affect the reproduction-related genes tested.

## THE EFFECT OF ZINC ON SURVIVAL AND REPRODUCTION DURING METAL STRESS

### Lethality tests and establishment of the LC value

To examine the possible protective properties of Zn on survival, 30 nematodes were exposed to 0.1 mM Zn in addition to the metal exposure and viability was scored after 24 hours (Fig. 3). A significant decrease in mortality of *C. elegans* was observed when coadministering Zn to As ( $p < 0.01$ ), Cd ( $p < 0.05$ ), and Cu ( $p < 0.01$ ). Even with the addition of Zn, almost all worms died at a 10 mM concentration of As, Cd, and Cu, indicating that Zn was not able to protect the worms from metal toxicity anymore. The LC50 values were 7.06 mM (6.35 mM – 7.82 mM) Cd+Zn, 6.62 mM (5.75 mM – 7.82 mM) As+Zn, 6.26 mM (5.71 mM – 7.09 mM) Cu+Zn, and 10.17 mM



**Fig. 4 Gene expression profile in *C. elegans* after metal exposure.** (A) Oxidative stress-related and (B) reproduction-related gene expression normalized using actin mRNA. Some changes are seen after As, Cd, Cu or Ni exposure for 12 h, whether or not, with or without the addition of zinc. *Sod2* and *ape1* are two genes associated with oxidative stress that show no expression changes for any of the treatments. The reproduction-related genes, except for *gld2* and *rog4*, do not show any gene expression changes after metal exposure as well. All values represent means;  $n = 6$ . \* $p < 0.05$  vs. control; \*\* $p < 0.01$  vs. control.

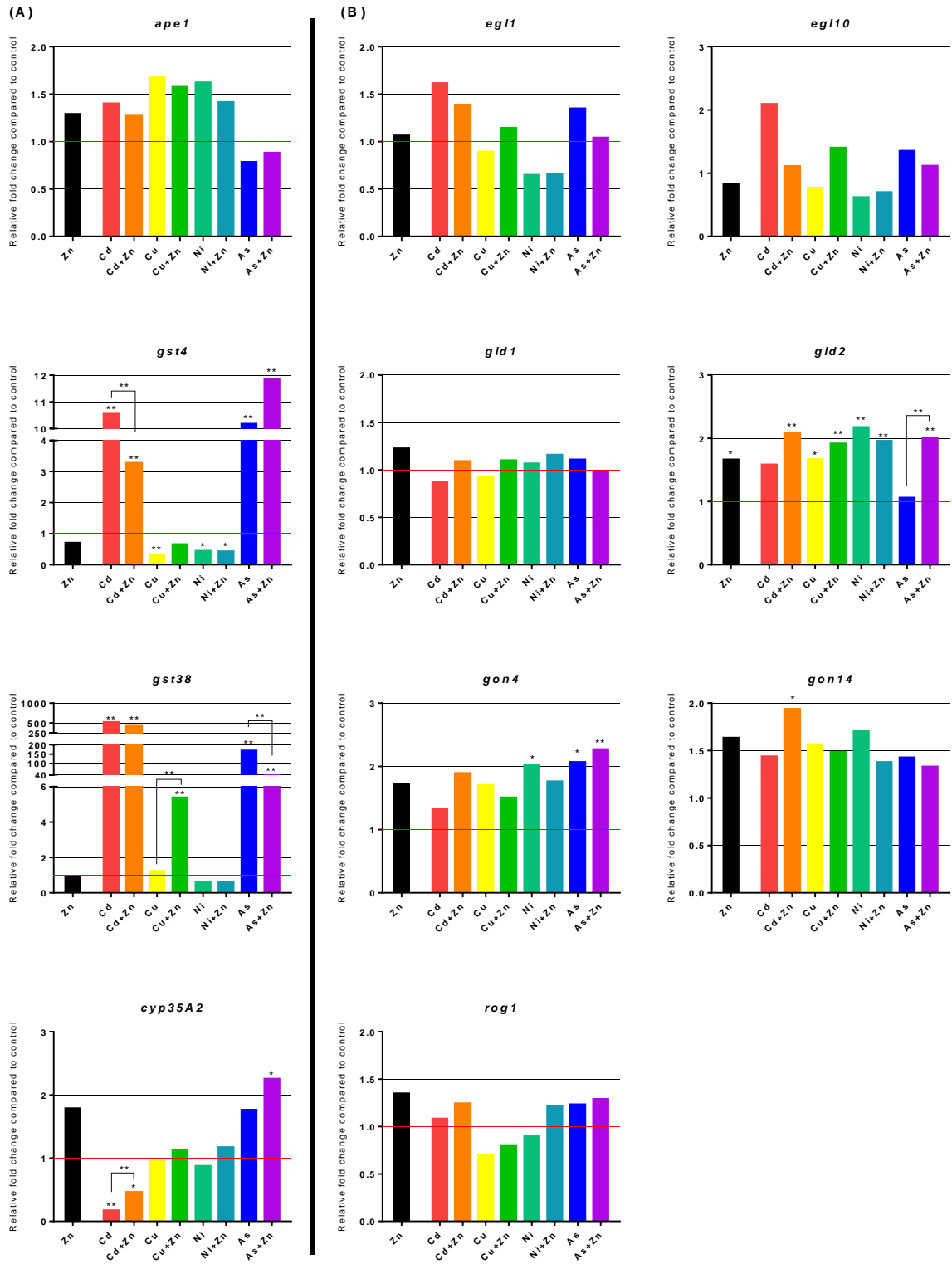


Fig. 4 Continued.

(8.70 mM – 12.70 mM) Ni+Zn. These data suggest that Zn is able to protect nematodes from the toxic effects of metals, but the noxious effects are insurmountable beyond a certain concentration.

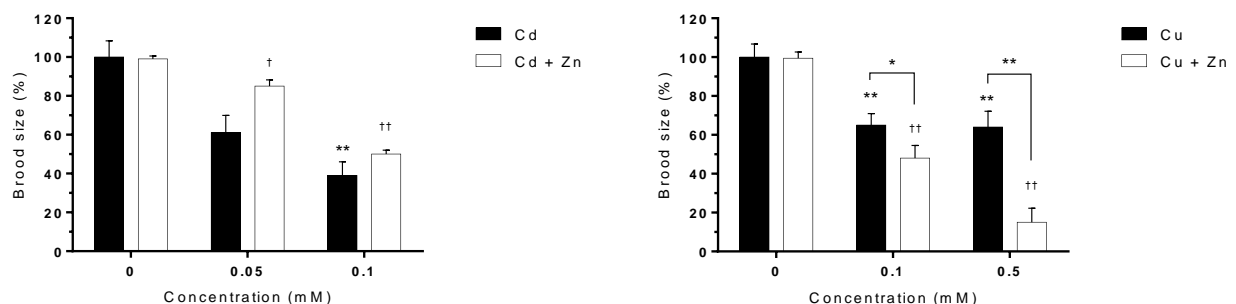
### ***Stress to the reproductive system: physiological level***

Next, the effect of Zn on the reproduction of *C. elegans* was tested. Because the previous study showed significant brood size changes for Cd and Cu, only these two metals were used in this experiment (Fig. 2B). The addition of Zn to Cd did not show significant changes in brood size, whereas the addition of Zn to Cu, rather unexpected, decreased the brood size of 0.1 mM Cu ( $p < 0.05$ ) and 0.5 mM Cu ( $p < 0.01$ ) (Fig. 5). However, a general increase in brood size could be observed when adding Zn to Cd. Zn did not seem to affect the brood size as expected, because no increase in the amount of eggs could be found after Zn supplementation.

### ***Stress to the reproductive system: molecular level***

Lastly, it was studied if Zn can also introduce changes at the molecular level of the nematodes compared to the experiment conducted earlier. The same stress-related and reproduction-related genes as in the previous experiment were considered: heat shock protein (*hsp70*, *hsp16.1*, *hsp16.2* and *hsp16.48*), metallothionein (*mtl1* and *mtl2*), catalase (*ctl1* and *ctl2*), superoxide dismutase (*sod2*, *sod3*, *sod4*), apoptosis enhancer (*ape1*), glutathione S-transferase (*gst4* and *gst38*), cytochrome p450 family protein 35A2 (*cyp35A2*), egg-laying defective (*elg1* and *elg10*), defective in germline development (*gld1* and *gld2*), abnormal gonad development (*gon4* and *gon14*), and Ras activating factor in development of germline (*rog1*). A comparison between each metal only and the metal along with Zn was done (Fig. 4).

All of the heat shock proteins and *gst4* were significantly upregulated after Zn addition to Cd (*hsp70* 142.8 folds, *hsp16.1* 94.7 folds, *hsp16.2* 51.8 folds, *hsp16.48* 57.5 folds, *gst4* 3.3 folds;  $p < 0.01$ ). However, a tremendous decrease in expression was observed when compared with the metal (*hsp70*, *hsp16.2*, and *hsp16.48*  $p < 0.05$ ; *hsp16.1* and *gst4*  $p < 0.01$ ) (Fig. 4A). In contrast, the transcript levels of the genes *mtl1* (110.4 folds,  $p < 0.01$ ), *mtl2* (8.6 folds,  $p < 0.01$ ), *ctl1* (2.4 folds,  $p < 0.01$ ), and *gst38* (441.1 folds,  $p < 0.01$ ) also remained upregulated. However, compared to Cd Zn addition had no differences pointing out its protective capabilities that were seen at the level of the organism. *Cyp35A2* was the only gene showing a significant downregulation after Zn supplementation (2.2 folds,  $p < 0.05$ ), however, this suppression was less severe than for the nematodes exposed to Cd alone (Cd vs. Cd+Zn  $p < 0.01$ ). Although *gld2* (2.1 folds,  $p < 0.01$ ) and *gon14* (1.9 folds,  $p < 0.05$ ) were the only two reproduction-related genes showing some overexpression compared to



**Fig. 5** The effect of metals on reproduction of *C. elegans*. Brood size is defined as the number of progeny of the parental worm. Each column represents a different concentration of a metal with and without the addition of Zn, respectively. Zn addition significantly decreases brood size of the parental worms exposed to Cu, while it has no obvious effects compared to parental worms exposed to Cd. Single synchronized young adult hermaphrodites were transferred to 60 mm Petri plates at indicated metal concentrations. Worms were transferred to a fresh Petri plate and removed from the experiment after 24 and 48 h, respectively. Data are expressed as percent of control. All values represent means  $\pm$  SEM;  $n = 4$ . \* $p < 0.05$  vs. control; \*\* $p < 0.01$  vs. control; † $p < 0.05$  vs. 0.1 mM Zn; †† $p < 0.01$  vs. 0.1 mM Zn.

the control, no shift in expression was found compared to the metal exposure itself (Fig. 4B).

Almost the same was true for the expression profiles of the nematodes exposed to Cu. *Gld2* was a bit upregulated compared to the control (1.9 folds,  $p < 0.01$ ), but the expression was not different of that of Cu (Fig. 4B). In three cases Zn addition resulted in an upregulated gene expression pattern compared to only Cu exposure: *mtl1* ( $p < 0.01$ ), *mtl2* ( $p < 0.05$ ), and *gst38* ( $p < 0.01$ ). The degree of increase for these three genes was 64.2 folds for *mtl1* ( $p < 0.01$ ), 3.3 folds for *mtl2* ( $p < 0.05$ ), and 5.4 folds for *gst38* ( $p < 0.01$ ). The degree of increase was also important for *hsp70* (12.2 folds,  $p < 0.01$ ), *hsp16.1* (12.1 folds,  $p < 0.01$ ), *hsp16.48* (6.1 folds,  $p < 0.01$ ), *ctl1* (14.0 folds,  $p < 0.01$ ), *sod3* (3.9 folds decrease,  $p < 0.05$ ), and *sod4* (13.4 folds,  $p < 0.01$ ), but no difference in expression with the metal was found (Fig. 4A).

Most of the genes did not show any up- or downregulation when Zn was supplemented in addition with Ni. The degree of change for these genes was less than 1.8 folds than that of the control. *Mtl1* and *ctl1*, on the other hand, were the only two genes that showed a significant difference between Ni and Ni+Zn exposure (Fig. 4A). For *mtl1* there was an upregulation ( $p < 0.05$ ), whereas for *ctl1* there was a downregulation ( $p < 0.01$ ) compared to the expression after Ni exposure. The stress-related genes *hsp16.2* (2.2 folds,  $p < 0.01$ ) and *gst4* (2.3 folds,  $p < 0.05$ ), and the reproduction-related gene *gld2* (2.0 folds,  $p < 0.01$ ) were respectively down- and upregulated compared to the control, however, their expression was not different of that of Ni.

*Ctl1* (2.8 folds,  $p < 0.01$ ) and *gst38* (41.1 folds,  $p < 0.01$ ) were the only stress-related genes that showed a difference in expression compared to As exposure (*ctl1*  $p < 0.05$ , *gst38*  $p < 0.01$ ). *Gld2* (2.0 folds,  $p < 0.01$ ) was the only reproduction-related gene (As vs. As+Zn  $p < 0.01$ ) (Fig. 4). The gene expression pattern was also increased after Zn supplementation for *hsp70* (6.6 folds,  $p < 0.01$ ), *hsp16.1* (13.6 folds,  $p < 0.01$ ), *hsp16.48* (8.1 folds,  $p < 0.01$ ), *mtl1* (171.9 folds,  $p < 0.01$ ), *mtl2* (3.3 folds,  $p < 0.05$ ), *skn1* (2.2 folds decrease,  $p < 0.05$ ), *gst4* (11.9 folds,  $p < 0.01$ ), *cyp35A2* (2.3 folds,  $p < 0.05$ ), and *gon4* (2.3 folds,  $p < 0.01$ ) without having any effect when compared to As exposure (Fig. 4A).

## Discussion

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During their lifetime humans are exposed to numerous chemicals that can enter the body through ingestion, inhalation or absorption causing severe health effects. Elucidating the mechanisms of metal toxicity is of great importance since they take up the first three places in the list of all hazards, a ranking compiled by U.S. ATSDR. One of the numerous drawbacks of metals is that they interfere with the reproductive system in many organisms. Zn, although a metal, is found to have protective properties, however, these findings have not been addressed in invertebrates. In this current study, the metals As, Cd, Cu, and Ni were chosen as chemical stressors to evaluate the potential protective effect of zinc on reproduction. The nematode *C. elegans* was used as a model organism and toxicity was assessed physiologically (mortality and egg-laying) and molecularly (transcript levels of both stress-related and reproduction-related genes).

### ***Metal exposure decreases the survival rate of C. elegans***

Previous studies demonstrated the suitability of *C. elegans* for the evaluation of lethal toxicity from environmental stressors including metal exposure<sup>[59, 75, 76]</sup>. To assess the toxicity level of the nematode *C. elegans* to As, Cd, Cu, and Ni dose-response curves were generated and 10%, 50%, and 90% lethal concentrations were calculated (Table 1). The order of chronic toxicity (LC50s) observed for *C. elegans* after 24 hours of exposure was as follows: Cu > As > Ni > Cd. This lethal toxicity manner agrees to previous reports<sup>[76-81]</sup>. However, these LC50s in units of mass (mg/l) were much higher compared with these reports. These differences can probably be attributed to technical variations (e.g. counting, tested medium, food source), sample size differences or sensitivity differences of the worms. When comparing the chronic toxicity of one metal to another using LC50 in atomic mass units (molar) for concentration, Cd was more toxic than Ni: Cu > As > Cd > Ni. In literature Cd has been described as a metal exhibiting a high level of tolerance compared to other metals among nematode species, implying that these organisms possess efficient defense mechanisms preventing Cd-related damage<sup>[79, 80, 82]</sup>. Rapid excretion, vesicles or specific proteins are potential mechanisms protecting against Cd toxicity in *C. elegans*<sup>[83, 84]</sup>. It seems that the mechanisms that were protecting the organisms against Cd-related damage during the first 24 hours were no longer capable of doing so after 48 hours. The 48 and 72 hours LC50s remain constant regardless of the units used: Cd > Cu > Ni > As. The order of toxicity observed in the present study is in accordance with the 96 hours LC50 data reported previously by Williams and Dusenbery (1990)<sup>[76]</sup>. Concisely, nematodes are more sensitive to metals when subjected to increased doses and/or when the duration of exposure to the toxicant is prolonged.

### ***Zinc increases the survival rate of C. elegans during metal stress***

Zn was administered to investigate its antioxidative properties in *C. elegans*. The addition of Zn to As, Cd, and Cu showed a significant decrease in mortality rate, whereas it had no effect on Ni (Fig. 3). The LC50 order was the same as above, with Cu and Ni respectively as the most and least toxic chemicals. There are a number of potential mechanisms that can explain the increased survival rate of the nematodes after Zn supplementation: (1) Zn competitively competes with the metals, especially Cu and Cd, for binding to the membrane transporters (e.g. CTR1 and ZIP), reducing the cellular uptake of these metals; (2) When the buffering capacity of the cell is exceeded, free Zn activates several protective mechanisms such as downregulation of Zn uptake transporters (ZIP) thereby also reducing the uptake of Cd ions, (3) induction of zinc efflux transporters (CDF1 and CDF2) and (4) induction of metal binding proteins (MT and PC)<sup>[85, 86]</sup>. Even when uptake of the metals into the cell took place, the metal binding proteins can bind and sequester them. The proposed mechanisms all have in common to decrease the oxidative stress response, which is detrimental for the normal function of the cells. However, beyond a certain metal exposure concentration (~10 mM As/Cd/Cu) Zn was not able to retain its protective effects. An elevation of the intracellular metal concentration probably triggered an irreversible oxidative stress response that the cell can no longer cope with. Taken together, Zn seemed to provide protection after metal



poisoning in *C. elegans*, but the mechanisms used to detoxify are rather unclear and need to be studied in more detail.

### ***Cadmium and copper affect the brood size***

The measurement of the brood size is a reflection of the reproductive capacity of the nematodes. Previous studies conducted by Swain *et al.* (2004), Wang and Wang (2008), and Wang *et al.* (2007) reported a remarkable toxicity on the brood size of the nematodes respectively after Cd, Ni, and As exposure<sup>[66-68]</sup>. In contrast, in this study the effect of the metals on the reproduction of the nematodes was inconclusive. Cd and Cu exposure led to a decrease in brood size, whereas As and Ni exposure had no effect (Fig. 2B). One possible explanation for the differences with previous reports is the sample size (*n*). In this study a pool of four worms for each exposure condition was monitored, opposed to 9-15 worms in the other studies mentioned above. A smaller sample size generally leads to a parameter estimate with larger variances within the tested groups, which in turn cause a larger difference that is needed to achieve a certain margin of error ( $\alpha$ ) of significance. A post-hoc power analysis was performed when no significant differences were found between the control group and the metal-exposed group (Supplemental data A). The power analysis showed insufficient power<sup>1</sup> for all tested groups, false negative results can therefore not be excluded. To increase the power of the study more individuals per group should be tested, but this was not feasible within the time frame of this study. The alteration in brood size of the nematodes exposed to Cd or Cu possibly indicates that essential reproductive processes are not resistant to the effects of Cd or Cu, whereas those processes are resistant upon As or Ni exposure. Arsenic and Ni may be excreted at a high rate, decreasing the toxic effects. Following toxicant exposure stress factors or detoxification processes may also be induced, allowing *C. elegans* to cope with the metals after the initial assault much more efficiently, compared to mammalian systems<sup>[87]</sup>. Additionally, the *C. elegans* reproductive system may be less sensitive to As and Ni toxicity. Another possible explanation for the reduced brood size after Cd and Cu exposure is the formation of worm bags within the parental body. This internal egg hatching, referred to as “worm bagging”, can be induced by exposure to toxic compounds and bacteria and is a result of a defective in egg-laying<sup>[88, 89]</sup>.

But the metal exposure was related to a 100% hatching probability after 48 hours suggesting that once an egg was laid nothing could stop it from hatching (Fig. 2A). That the progeny was able to writhe to move out of its egg indicates the development of an advanced motor system during the embryonic stages of life. The hatched progeny within 24 hours after exposure with Cu was less compared to control, which could indicate that the development of their motor system is slightly delayed.

In summary, these experimental findings suggest an egg-laying muscle defect, an egg production defect or a fertilization defect of the parental worm as a result of Cd and Cu exposure.

### ***Zinc alters brood size after copper coadministration***

The hypothesis of this study states that Zn reduces the adverse effects of metals on the reproductive system. In this respect, Zn was only coadministered to the metals Cd and Cu (Fig. 5). In accordance with the results obtained from the lethality tests, a similar protective effect of Zn was expected on the reproductive system of the stressed nematodes. Although, Cd coadministration just showed a general increase in reproductive capacity, coadministration of Cu showed a significant decrease. Again, the sample size (*n*) can possibly be held responsible for the lack of significant Cd group differences. To investigate if the experiment achieved sufficient

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<sup>1</sup> Insufficient power = no chance to detect an effect, that perhaps might be present

power, a post-hoc power analysis was performed between the animals exposed to Cd and the animals coadministered with Zn ([Supplemental data B](#)). The tested groups (0.05 mM Cd vs. Cd+Zn and 0.1 mM Cd vs. Cd+Zn) both showed insufficient power, which increases the chance of making a type II error (false negatives). In summary, these data indicate that Zn has no protective effect on the reproductive system of the organism on the physiological level.

### ***Nematodes exposed to metals exhibit an altered stress-related gene expression***

Before the effect of Zn cotreatment on the gene expression profile could be identified, the nematodes were exposed to the heavy metals and the gene expression changes of several stress-related and reproduction-related genes were examined ([Fig. 4](#)).

First of all, the elevated levels of *mtl1* mRNA after Zn administration should be explained. MTs play a role in both metal detoxification and homeostasis. They bind both metals of physiological importance, as well as xenobiotics<sup>[17, 20]</sup>. *Mtl1* is upregulated as a result of the increased exposure to Zn. The generated MT proteins bind free intracellular Zn thereby maintaining the homeostatic balance.

The upregulation of the *ctl*, *gst*, and *hsp* genes after Cd exposure are an indication that the nematodes suffer from oxidative stress. The extracellular located SOD4 enzyme was the only member of the SOD family that was transcriptionally upregulated in response to Cd exposure, indicating that mitochondrial and cytosolic ROS amounts are kept under control within acceptable limits through other mechanisms. Together with the other antioxidative genes described above MTs are such proteins that can bind and sequester Cd<sup>2+</sup> ions. Real-time PCR analysis confirmed the upregulation of both *mtl* genes at the transcriptional level. These findings differ from a previous report conducted by Roh *et al.* (2006) where no significant changes were observed for the *hsp16.48* and *mtl1* genes<sup>[80]</sup>. Swain *et al.* (2004) on the other hand confirmed our findings by stating that Cd is a potent inducer of *mtl1* and *mtl2* mRNA levels<sup>[66]</sup>. Further, Roh *et al.* (2006) showed a 7.9 fold increase in *ape1* gene expression, whereas in this study an increase of only 1.4 folds compared to control ( $p > 0.05$ ) was observed. This non-significant minor increase of an apoptosis inhibitor again is an indication that Cd exposure is counteracted by several mechanisms before triggering apoptotic effects. In this study, a large transcriptional decrease was seen for the *cyp35A2* gene during Cd exposure compared to control. Roh *et al.* (2006) on the other hand showed a 4.7 fold increase for *cyp35A2* after exposure to ~0.05 mM CdCl<sub>2</sub> (8.5 mg/l)<sup>[80]</sup>. A sensitive response at the molecular level may contribute to the organism-level resistance, which may be translated into the high LC50 (Cu > As > Ni > Cd). Although, the increase in many stress-related gene expressions occurred concomitantly with the deterioration of the reproduction at the physiological level ([Fig. 2B](#)). Taken together, it was found that the stress-responsive genes, but not the reproduction-related genes were responsible for the changes at the level of the organism after Cd exposure.

The responses of the stress-related genes to Cu were not as intense as their responses to Cd exposure. The mitochondrial located SOD3 and extracellular located SOD4 enzymes were respectively down- and upregulated following Cu exposure. The transcriptional upregulation of *sod4* is a result of the elevated extracellular Cu concentration. The increased SOD4 enzyme production ensures a reduction in the extracellular Cu concentration because Cu acts as a cofactor of SOD4. This extracellular concentration reduction probably has an indirect effect on the gene expression profiles of the other stress-responsive genes. The gene expression profile revealed that the Cu<sup>+</sup> ions were not predominantly bound to MTs as were Cd<sup>2+</sup> ions. It can also be concluded from the *gst38* steady-state gene level and the significant downregulation of *gst4* that Cu also is not predominantly bound to GSH. These findings are in contrast with literature. Martinez-Finley *et al.* (2012) stated that MT and GSH have high affinity for Cu<sup>[17]</sup>. It is supposed that Cu mainly stays outside of the cells, by binding to SOD4, thereby avoiding the upregulation of the genes that encode the cytosolic proteins. *Ctl1* on the other hand is upregulated, which implies that H<sub>2</sub>O<sub>2</sub> is present in the cytosol of the cell. It can be assumed that this is caused by: 1) Transport of H<sub>2</sub>O<sub>2</sub>, caused by SOD4, from the extracellular matrix into the cell; 2) The catalization of superoxide into O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> by cytosolic SOD1 or SOD5. It can be hypothesized that SOD1 and SOD5 also

play a role in the increase in the amount of cytosolic H<sub>2</sub>O<sub>2</sub> after Cu exposure, because of their affinity for Cu binding. *Hsp70*, *hsp16.1* and *hsp16.48* were significantly upregulated compared to control. Although there was no significance detected for *hsp16.2*, it was three times more upregulated than control. This was probably because of the large variances within this tested group. The opposite was seen for the reproduction-related *gld2* gene, where the significance was probably caused by a small variance. However, this increase in expression was less than twofold and can therefore be neglected. Summarized, it seems that most of the Cu is bound extracellularly by SOD4 thereby decreasing the response of the other stress-related genes. This decrease in stress-related gene responses can also be seen on the reproductive system at the physiological level (Fig. 2B). Like Cd, Cu showed a decrease in brood size, but this was less severe than in the nematodes exposed to Cd. Again, the reproduction-related genes did not seem to have an effect on reproduction.

In the previous experiment with Ni no changes in brood size were observed compared with control (Fig. 2B). It was proposed that high rate excretion of Ni might underlie these findings. Therefore it is supposed that the nematodes experience almost no stress. This thought is confirmed when considering the stress-related gene expression profiles. Only upregulation of the *ctl1* gene was observed, indicating a minor increase in cytosolic ROS amount. *Gld2* and *gon4*, both reproduction-related genes, showed some overexpression of mRNA compared to the steady-state levels. *Gon4* is required for gonadogenesis and upregulation of GLD2 cytoplasmic poly(A) RNA polymerase reinforces the decision to enter meiosis from the mitotic cell cycle and is also required for progression through the meiotic prophase during oogenesis and spermatogenesis. It is assumed that both gene expressions were upregulated as a counteract for adverse Ni effects on the reproductive system, thereby leading to unchanged brood size compared with control worms.

As for Ni, no changes in brood size were observed after As exposure (Fig. 2B). However, several gene expression profiles were significantly upregulated. Three out of four tested heat shock proteins showed a significant upregulation of their mRNA levels indicating that the cells suffer from metal stress. The fact that all *ctl* and *sod* gene expressions were not significantly altered indicates that there was no increased ROS production during As exposure. This ROS balance is probably maintained by the active binding of intracellular As<sup>3+</sup> ions to MTs and GSHs, seen by an upregulation in transcription of both MT proteins and GSH peptides. Berman *et al.* (2001) suggested the involvement of the p38 mitogen-activated protein kinase (MAPK) pathway in controlling the arsenite stress response<sup>[90]</sup>. Inoue *et al.* (2004) discovered the translocation of SKN1 into the nucleus in response to phosphorylation of p38 map kinase family (PMK1). SKN1 in turn activates the transcription of the arsenite inducible protein 1 (*aip1*) gene encoding a protein which is necessary for the protection of the cells from arsenite toxicity<sup>[91]</sup>. The significant repression of *skn1* mRNA levels seen in this study supports the idea that almost no oxidative stress in the cells is induced.

In summary, it looks like there is a relationship between the brood size of the nematodes and the expression of their stress-related genes. Especially the increased formation of ROS seems to have a negative impact on the brood size. The reproduction-related genes showed almost no differences in expression compared to control, suggesting that there is no problem with embryogenesis (spermatogenesis, oogenesis), but parental worms rather reduce the brood size until environmental conditions become more favorable.

### ***Zn coadministration has alternating effects on the gene expression profile***

The hypothesis of this study also states that Zn cotreatment reduces the oxidative stress caused by metal exposure. In order to test this hypothesis Zn was coadministered and real-time PCR analysis was performed of both stress- and reproduction-responsive genes (Fig. 4).

Cadmium cotreatment resulted in diminished responses of the *hsp* genes compared to Cd exposure. Though, the genes were still highly upregulated compared to steady-state levels. *Gst4* and *cyp35A2* mRNA levels were respectively decreased and increased after Zn addition compared to Cd exposure alone. In contrast to this findings, Bay *et al.* (1996) and Cabré *et al.* (2001) both found that Zn administration reduced the cytochrome

P450 content<sup>[92, 93]</sup>. The mechanism by which Zn increases the mRNA levels of *cyp35A2* compared to Cd is unclear. These alterations in gene expressions were accompanied by a general increase in brood size on the physiological level (Fig. 5). According to the gene expression changes it can be assumed that the oxidative stress inside the cells is reduced. It seems that Zn reduces this stress by reducing the intracellular Cd concentration, by inhibiting the uptake of Cd into the cells or inducing of efflux transporters. This theory is supported by the general, though large, decrease in expression of both *mtl* genes compared to Cd.

At the physiological level there was a significant decrease in brood size compared with only Cu-treated animals (Fig. 5). As mentioned earlier, Swain *et al.* (2004) reported that both Cu and Zn were poor inducers of the transcriptional *mtl* response<sup>[66]</sup>. However, the coadministration of these two metals resulted in a significant upregulation of both *mtl* and *gst38* gene expression levels. Because there is a significant decrease in brood size, referring to increased oxidative stress, it is hypothesized that the upregulation in *mtl* and *gst38* mRNA levels will not be seen at the protein levels, due to post-transcriptional modifications.

Zn coadministration with Ni led to a significant decrease and increase respectively in *ctl1* and *mtl1* gene expression compared with Ni-exposed animals. The *ctl1* gene transcription level was comparable with that of control ( $p > 0.05$ ). This implies that the ROS amounts were reduced to normal levels after Zn coadministration. The increase in *mtl1* transcription is probably solely due to Zn administration, because Zn treatment only resulted in increased *mtl* mRNA levels. Although no experiments were performed to measure the brood size, it can be hypothesized that brood size from Zn coadministered nematodes will not differ from control worms.

An upregulation in *ctl1* and *gld2*, and a downregulation, though still upregulated compared to control, in *gst38* mRNA levels was observed during Zn cotreatment compared with only As exposure. The upregulation of *ctl1* after As-Zn exposure compared to As exposure means that there is slightly more ROS present in the cytosol. What causes the downregulation of *gst38* mRNA levels after Zn cotreatment compared with As exposure is rather unclear, and needs more research. Overall, the cells are subjected to oxidative stress, what is seen by the elevation of some *hsp*, *mtl*, *ctl*, and *gst* mRNA levels. Therefore it is supposed that Zn cotreatment will have no effect or a small decline in the brood size compared to As exposure. The upregulated *gld2* gene expression profile after Zn cotreatment compared to As exposure indicates increased embryo development. But this has no effect on the brood size of the worms.

In summary, Zn coadministration had varying effects on the gene expression profiles. A decrease of oxidative stress was seen for Cd and Ni exposure cotreated with Zn. In contrast, an increase was seen for Cu and As. The stress responses for Cd and Cu were inversely related to the observed brood sizes. It can therefore be assumed that this will be the same for Ni and As, but further studies are needed to confirm this theory. The hypothesis of the protective effect of Zn on reproduction by reducing oxidative stress appears invalid in this study, however, further study may still prove its protective effects.



## Conclusion

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The goal of this study was to investigate the effect of Zn on the reproductive ability of *C. elegans* after exposure to several metals. Heavy metals have been addressed as toxic to different organ systems, including the reproductive system. During their evolution, organisms have developed several detoxification systems such as GSH, MTs, HSPs, and transporters to defend themselves. Zn is a trace element with well-known antioxidant properties that is tightly controlled. It is hypothesized that Zn reduces metal-induced oxidative stress, resulting in beneficial effects on the reproductive system.

Nematodes were treated with As, Cd, Cu, or Ni to assess lethal toxicity after 24, 48, and 72 hours. It was found that the nematodes were more sensitive when subjected to increased doses and/or when the duration of exposure to the toxicant was prolonged. Zn cotreatment showed a significant increase in survival of *C. elegans* for all metals but Ni. Several mechanisms including reduced cellular uptake, downregulation of influx transporters, induction of efflux transporters, and induction of metal binding proteins can underlie these findings. This study only focused on the expression profile of stress- and reproduction-related genes. So, future research is needed to decipher the exact working mechanisms of Zn in invertebrates.

The next experiment in this study determined the number of offspring after metal exposure. Cd and Cu showed decreased brood sizes, whereas As and Ni were not different from the control worms. In contrast, in literature all four of these metals were shown to decrease the brood size of *C. elegans*. Repetition of this experiment in the future will give some more insight in the toxicity of As and Ni. Because it was hypothesized that Zn increases the brood size compared with metal exposure, Zn was only coadministered with Cd and Cu. The findings of this experiment were surprising. Zn did not have an effect on Cd, but had a negative impact on Cu-treated animals. Again, repetition is needed to provide more insight on the effects of Zn on reproduction at the physiological level. By analogy with the Cu-Zn cotreatment, experiments on the brood size with As and Ni can be conducted to determine the effect of Zn.

Lastly, the gene expression profiles of several stress- and reproduction-related genes were investigated to assess the effects of metals on the molecular level. A relationship was found between the stress-related genes and the changes on the reproductive system at the physiological level. The increased formation of ROS seemed to be the key player in the reduction of the brood size of the nematodes. The upregulation of the gene expression of ROS catalyzing enzymes in Cd and Cu exposed animals were related with a decrease in brood size, whereas the steady-state levels of these genes seen in As and Ni exposed animals were related to normal brood sizes. Zn cotreatment had different outcomes on the gene expression profiles. For Cd and Ni, cotreated with Zn, there was a decrease in oxidative stress observed. For Cu and As on the other hand increased stress responses are seen. The brood sizes of Cd-Zn- and Cu-Zn-treated animals were inversely related to the observed stress responses, the more stress the less progeny and vice versa. It can therefore be assumed that the brood sizes of Ni-Zn- and As-Zn-treated animals respectively will be equal or increased and equal or decreased compared with only metal exposure.

To conclude, Zn can possibly be used as a treatment of certain metal toxicities in humans. But further studies are needed to confirm its potential protective effects on the reproductive system.



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# Supplemental data

## Supplemental data A

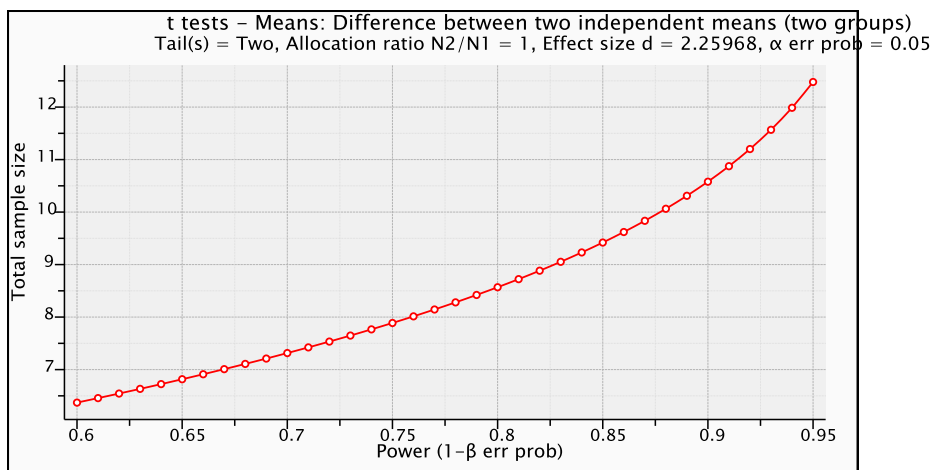
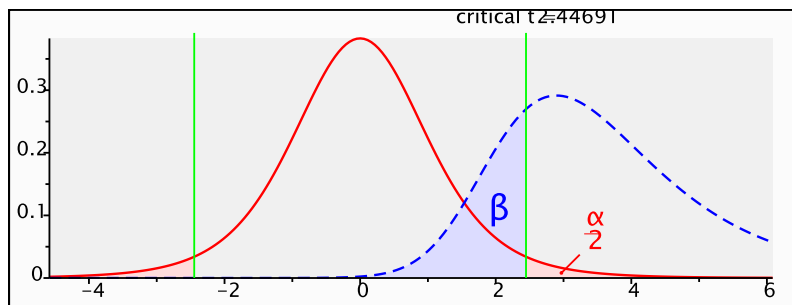
### 0.1 mM Cadmium

t tests – Means: Difference between two independent means (two groups)

Analysis: Post hoc: Compute achieved power

Input: Tail(s) = Two  
Effect size d = 2.2596791  
 $\alpha$  err prob = 0.05  
Sample size group 1 = 4  
Sample size group 2 = 4

Output: Noncentrality parameter  $\delta$  = 3.195669  
Critical t = 2.446912  
Df = 6  
Power ( $1 - \beta$  err prob) = 0.758923



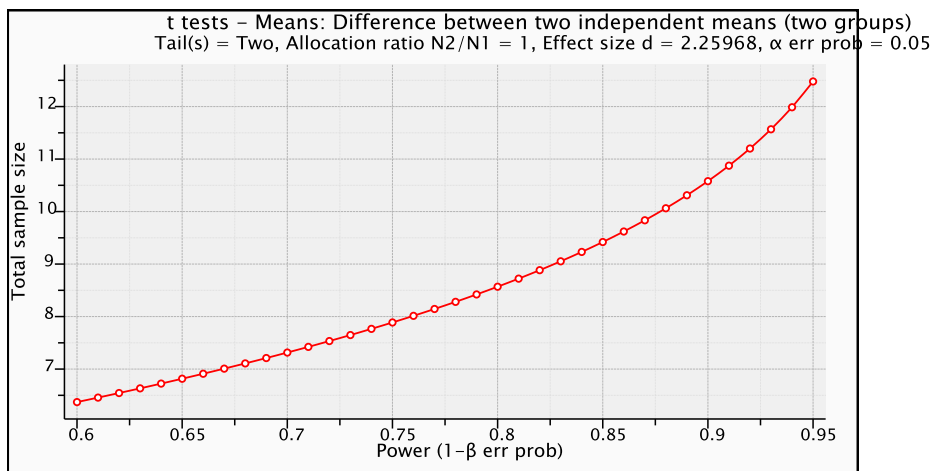
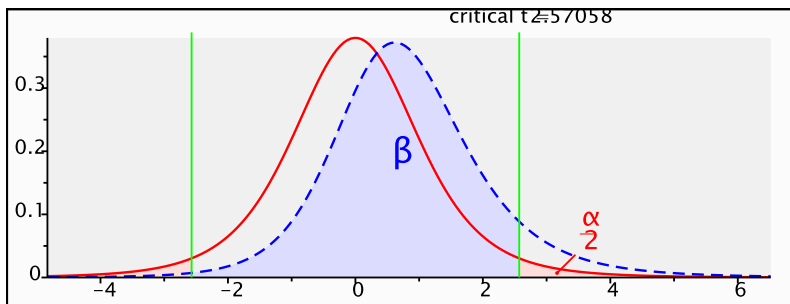
**0.1 mM Arsenic**

**t tests – Means: Difference between two independent means (two groups)**

**Analysis:** Post hoc: Compute achieved power

**Input:** Tail(s) = Two  
 Effect size d = 0.5382521  
 $\alpha$  err prob = 0.05  
 Sample size group 1 = 3  
 Sample size group 2 = 4

**Output:** Noncentrality parameter  $\delta$  = 0.704737  
 Critical t = 2.570582  
 Df = 5  
 Power ( $1-\beta$  err prob) = 0.089306



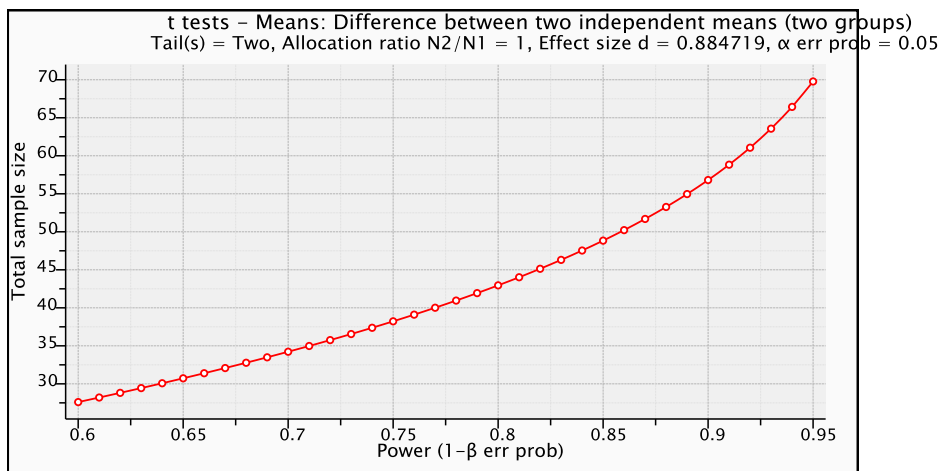
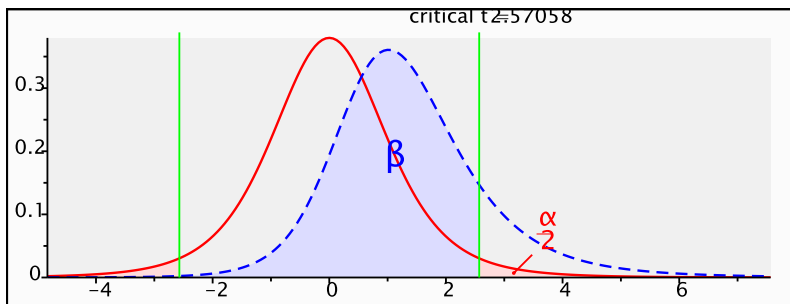
### 1 mM Arsenic

**t tests** – Means: Difference between two independent means (two groups)

**Analysis:** Post hoc: Compute achieved power

**Input:** Tail(s) = Two  
Effect size d = 0.8847189  
 $\alpha$  err prob = 0.05  
Sample size group 1 = 3  
Sample size group 2 = 4

**Output:** Noncentrality parameter  $\delta$  = 1.158369  
Critical t = 2.570582  
Df = 5  
Power ( $1-\beta$  err prob) = 0.157804



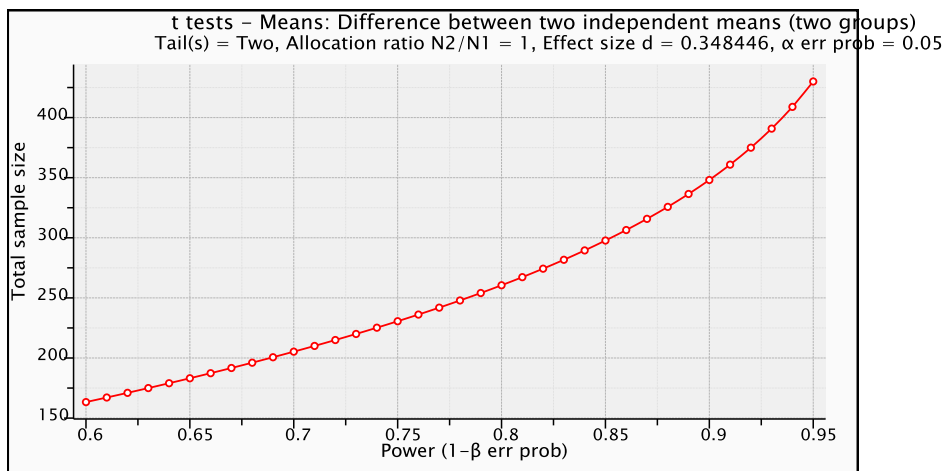
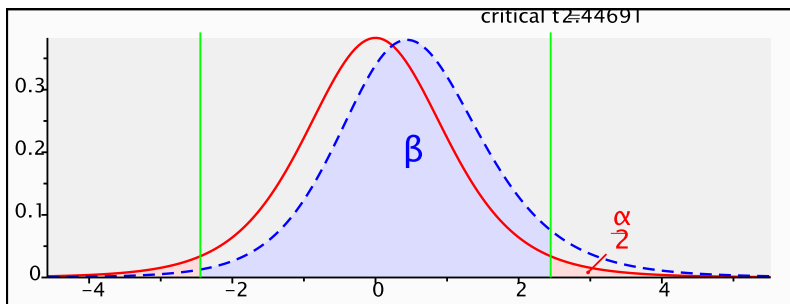
**0.05 mM Nickel**

**t tests – Means: Difference between two independent means (two groups)**

**Analysis:** Post hoc: Compute achieved power

**Input:** Tail(s) = Two  
Effect size d = 0.3484455  
 $\alpha$  err prob = 0.05  
Sample size group 1 = 4  
Sample size group 2 = 4

**Output:** Noncentrality parameter  $\delta$  = 0.492776  
Critical t = 2.446912  
Df = 6  
Power (1- $\beta$  err prob) = 0.070364



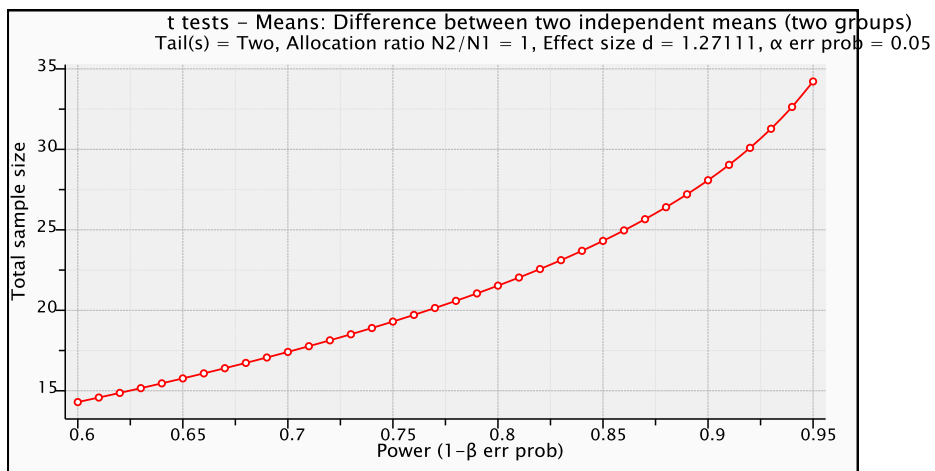
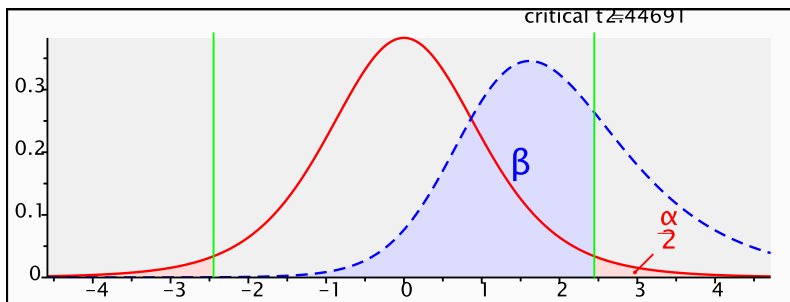
### 0.1 mM Nickel

**t tests** – Means: Difference between two independent means (two groups)

**Analysis:** Post hoc: Compute achieved power

**Input:** Tail(s) = Two  
Effect size d = 1.2711144  
 $\alpha$  err prob = 0.05  
Sample size group 1 = 4  
Sample size group 2 = 4

**Output:** Noncentrality parameter  $\delta$  = 1.797627  
Critical t = 2.446912  
Df = 6  
Power ( $1-\beta$  err prob) = 0.328533





## Supplemental data B

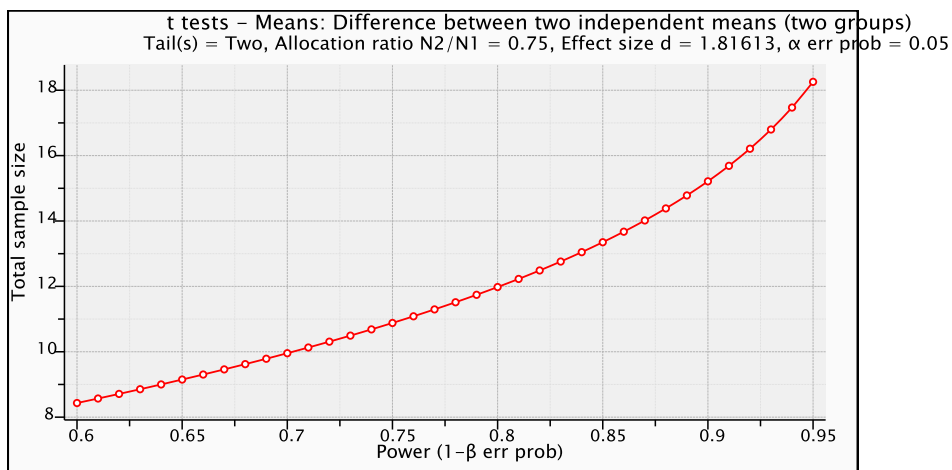
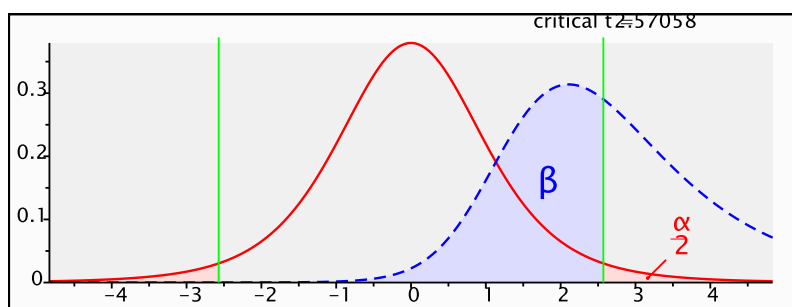
### 0.05 mM Cadmium + zinc

**t tests** – Means: Difference between two independent means (two groups)

**Analysis:** Post hoc: Compute achieved power

**Input:** Tail(s) = Two  
Effect size d = 1.8161290  
 $\alpha$  err prob = 0.05  
Sample size group 1 = 4  
Sample size group 2 = 3

**Output:** Noncentrality parameter  $\delta$  = 2.377871  
Critical t = 2.570582  
Df = 5  
Power ( $1 - \beta$  err prob) = 0.484440



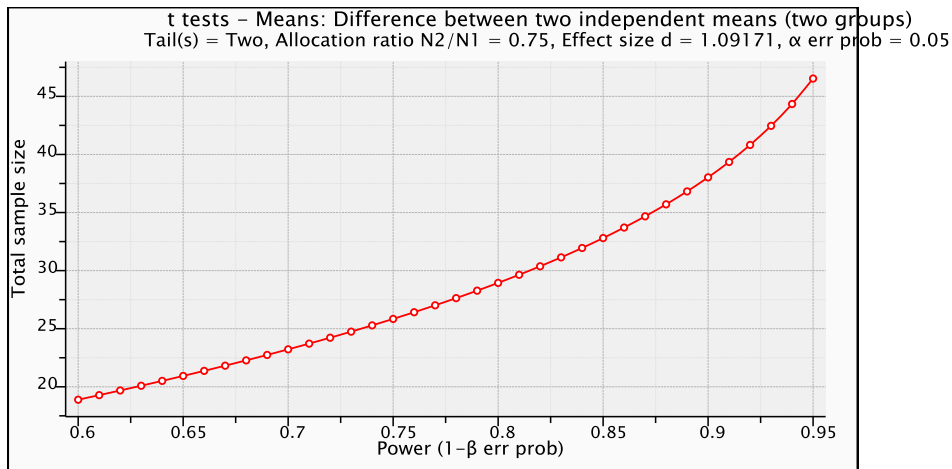
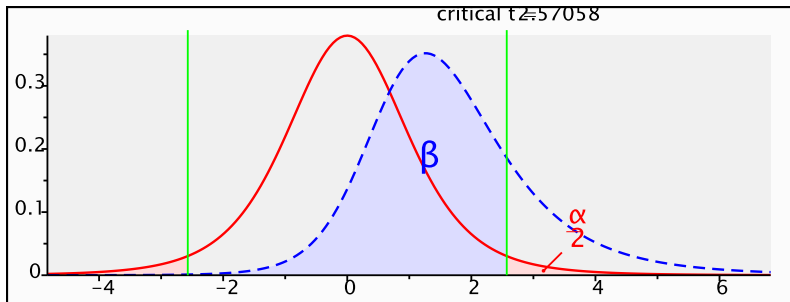
**0.1 mM Cadmium + zinc**

**t tests – Means: Difference between two independent means (two groups)**

**Analysis:** Post hoc: Compute achieved power

**Input:** Tail(s) = Two  
 Effect size d = 1.0917081  
 $\alpha$  err prob = 0.05  
 Sample size group 1 = 4  
 Sample size group 2 = 3

**Output:** Noncentrality parameter  $\delta$  = 1.429381  
 Critical t = 2.570582  
 Df = 5  
 Power ( $1 - \beta$  err prob) = 0.214913



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Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:

**Protective effect of Zn<sup>2+</sup> on heavy metal-induced reproductive toxicity**

Richting: **master in de biomedische wetenschappen-milieu en gezondheid**

Jaar: **2014**

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