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Combined exposure to methylmercury and manganese during L1 larval stage causes motor dysfunction, cholinergic and monoaminergic up-regulation and oxidative stress in L4 *Caenorhabditis elegans*

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Abstract

Humans are exposed simultaneously to a variety of neurotoxic agents, including manganese (Mn) and methylmercury (MeHg). Therefore, the study of combined exposures to toxicants is timely. This work aimed to study changes in cholinergic system focusing on acetylcholinesterase (*ace-2*), monoaminergic system focusing on vesicular monoamine transporter (VMAT, *cat-1*) expression, to address changes in antioxidant enzymatic systems, namely, the expression of superoxide dismutase (*sod-3 and sod-4*) and catalase (*ctl-3*), as well as worm reproduction and locomotion. *C. elegans* in the L1 larval stage were exposed to Mn, MeHg or both. All analyses were done 24 h after the end of exposure, except for behavior and reproduction tests that were assessed in L4 larval stage worms. The values obtained for lethal dose 50% (LD_{50}) were 17.78 mM for Mn and 30.63 μM for MeHg. It was observed that body bends, pharyngeal pumping and brood size decreased in worms exposed to metals when undergoing combined exposures. Relative Mrna content of *ace-2,cat-1,sod- 3, sod-4 and ctl-3* was increased at the highest concentration of the interaction (50 mM Mn + 50 μM MeHg). Cholinergic degeneration was observed in all groups co-exposed to both metals. Notably, combined exposure to metals was more toxic to the worms than when exposed to a single metal.

Graphical Abstract:

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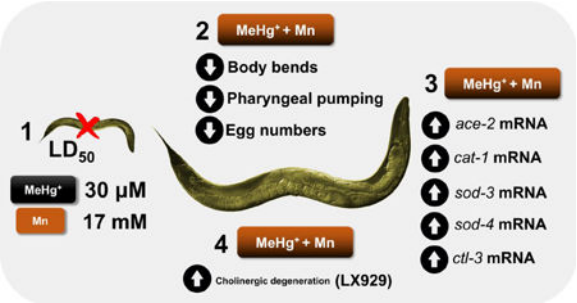
Conflicts of interest

There are no conflicts of interest to declare.



Overview of all the results obtained in this work. MeHg (methylmercury), Mn (manganese), LD50 (lethal dose that kills 50 % of the animals), *ace-2* (acetylcholinesterase-2), *cat-1* (Vesicular monoamine transporter/VMAT), *sod-3* (superoxide dismutase-3), *sod-4* (superoxide dismutase-4), *ctl-3* (catalase-1), LX929 (fluorescent strain to cholinergic neurons).

Keywords

metal interaction; development; C. *elegans*; behavioral tasks; neurodegeneration

1. Introduction

Concurrent human exposure to toxic heavy metals is a persistent health problem, mainly as it relates to long-lasting effects. In fact, exposure to multiple metals captures real-life scenarios and public health issues (Grandjean and Landrigan, 2014). Adverse effects of metals are known to occur during neurodevelopment and may cause impairments in the ability to perform simple or complex cognitive tasks. The mechanisms involved in neurotoxicity associated with simultaneous exposure to metals are unclear and they may result from additive/synergetic or antagonistic effects (Karri et al., 2016; Wang et al., 2017). Environmental disasters, such as recent waste deposit break in the city of Mariana, in Minas Gerais, Brazil (2015), where several toxic elements including MeHg and Mn were released together into the environment, may cause unknown long-term effects (Segura et al., 2016).

Recently, the effects of mixtures have been tested in *Caenorhabditis elegans* by Wang et al. (2017), addressing the effects of arsenic, copper and glyphosate on behavior and reproduction. In another study, Moyson et al. (2017) analyzed the effects of copper, cadmium and zinc on behavior and mortality in the nematode. *C. elegans* is an excellent model to study metal toxicity because of its high genetic homology to humans, low cost, well characterized phases of development, small size and sensitivity to different types of stress (Perendey and Williams, 2000; Leung et al., 2008). Grandjean & Landrigan (2014) identified chemicals classified as developmental neurotoxicants, among them are MeHg and Mn. Accordingly, these chemicals were chosen to assess the consequences of simultaneous exposures in the nematode *C. elegans*.

Mn is an essential metal for animals. It plays a vital role in several important physiological processes, including normal development, reproduction, immune response, metabolic rate and antioxidant defenses against cellular stressors (Chen et al., 2015; 2016). For example,

Mn acts as a cofactor for the enzyme Mn-superoxide dismutase (MnSOD). However, at high concentrations, it may induce neurotoxicity secondary to the generation of reactive oxygen species (Martinez-Finley et al., 2013). In humans, toxicity commonly occurs upon occupational exposure, food or water with elevated levels of Mn (Peres et al., 2016). Mn is a prominent risk factor for developing parkinsonism, and occupational exposure to high levels may cause a syndrome referred to as manganism, with symptoms closely resembling Parkinson's disease (PD) (Angeli et al., 2014). In *C. elegans* Mn can damage dopaminergic rather than GABAergic neurons (Chen et al., 2015; 2016). However little is known about the effect of Mn on the cholinergic system (Benedetto et al., 2010).

The distribution, toxicity and metabolism of mercury (Hg) is largely dependent on its chemical form (Clarkson & Magos, 2006). MeHg is particularly toxic in the early phases of development (Patel and Reynolds, 2013). Normally the main source of MeHg is by seafood ingestion due to water contamination from human activities (Clarkson and Magos, 2006). MeHg can affect calcium homeostasis, cause central nervous system (CNS) degeneration and impair normal neurodevelopment (Santos et al., 2016), generate reactive oxygen species and alter the activity of several important enzymes affecting metabolism (Martinez-Finley, 2013; Castoldi et al., 2008). Similar effects to those found in mammals have been shown in *C. elegans* (Helmcke et al., 2009; Caito et al., 2013). However, scarce data exist on the toxicological effects of MeHg on the cholinergic system in *C. elegans* (Helmcke et al., 2009; Arantes et al., 2016).

The cholinergic system is one of the oldest systems in the evolutionary pathway and its neurotransmitter acetylcholine (ACh) is widely distributed in living organisms. In *C. elegans* ACh is the major excitatory neurotransmitter at the neuromuscular junctions. More than a third of the cells in the *C. elegans* nervous system release ACh (Pereira et al., 2015). The mammalian enzymes that synthetizes ACh (choline acetyltransferase) and hydrolyze ACh (acetylcholinesterase), as well as transporters and receptors inherent to cholinerthegic system have all homologs in *C. elegans*. Cholinergic transmission is involved, directly or indirectly, in many *C. elegans* behaviors, including locomotion, egg laying and pharyngeal pumping (Rand, 2007).

C. elegans contains an endogenous monoaminergic signaling system including the monoamines serotonin (5-HT), tyramine (TA), octopamine (OA) and dopamine (DA). These are extremely important to nematode survival and adaptation, modulating an array of key behaviors including feeding, pharyngeal pumping, locomotion and egg laying (Duerr et al., 1999; Kominiecki et al., 2012; Donnelly et al., 2013). The loading of these molecules into synaptic vesicles is mediated by specific vesicular monoamine transporters (VMATs). Specifically, in *C. elegans* the *cat-1* gene encodes a vesicular monoamine transporter which is required for monoamine-dependent behaviors (Duerr et al., 1999).

Several studies have reported the toxic effects of MeHg (Arantes et al., 2016; Caito and Aschner, 2016; Rudgalvyte et al., 2017) or Mn (Benedetto et al., 2010; Chen et al., 2015; Peres et al., 2016) on *C. elegans.* It is known that co-exposure to metals may produce additive/synergetic effects by overlapping intrinsic mechanisms of action (Karri et al., 2016). Here, we addressed whether co-exposure to MeHg and Mn causes more pronounced effects

than single metal exposure. We assessed lethality, behavioral parameters, and expression of proteins related to the cholinergic, monoaminergic or the antioxidant enzymatic systems.

2. Material and methods

2.1. C. elegans maintenance

Strains used in this study: Bristol N2 (wild-type; WT), LX929 (*vsls48[unc-17::GFP*]), DA453 (*eat-2(ad453)II*), RB918 (*acr-16(ok789)V*), JD217 (*gar-3(vu78) V*), GG201 (*ace-2(g72) I; ace-1(p1000) X*) and PR1152 (*cha-1(p1152) IV*) as well as *Escherichia coli* OP50 and NA22 were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota, MN, USA. Nematodes were grown on Nematode Growth Medium (NGM) agar plates carrying a lawn of *E. coli* NA22 until synchronization and after maintained on agar plates carrying a lawn of *E. coli* OP50 for experimental analyzes (Benedetto et al., 2010). Synchronization of nematode cultures was achieved by bleaching treatment of gravid hermaphrodites (Lewis and Fleming, 1995), and the obtained eggs were allowed to hatch overnight in M9 buffer (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl and 1 mM $MgSO_4$) at 20°C.

The mutant strains studied were: PR1152 a mutant strain to choline acetyltranferase1 (*cha-1*), with depletion in the synthesis of ACh and reduced pharyngeal pumping; GG201, a mutant (substitution) of acetylcholinesterase 1 (*ace-1*) and 2 (*ace-2*), with decreased hydrolysis of ACh presenting slow movement; JD217, *gar-3* a mutant (substitution) with deficient pharyngeal movement dependent on muscarinic activation; RB918, *acr-16* a mutant (deletion) with decreased nicotinic muscle activity; and DA453, *eat-2* a mutant (substitution) presenting slow pharyngeal pumping and low feeding behavior.

2.2. Lethality

First, we determined a dose–response curve to evaluate MeHg (0–60 μM) and Mn lethality (0–100 mM) alone or in combination. After 30 min. exposure to MeHg, Mn or both (in M9 in the absence of *E. coli*) worms were washed three times with M9. Then, approximately 100 worms per treatment were transferred to NGM plates seeded with *E. coli* OP50. After 24 h, nematodes were scored as alive or dead independently of the development stage with a stereomicroscope. Non-responding animals to a mechanical stimulus with a platinum wire were considered dead. After obtaining LD_{50} values for MeHg and Mn, we selected a high dose, a low dose and one dose approximating these values for the co-exposure to MeHg and Mn. All LD_{50} experiments were repeated independently at least 4 times.

2.3. Reproduction tests

After the exposure to MeHg, Mn or both, at the L1 larvae stage, animals were placed on NGM plates with *E. coli* OP50 for a recovery period. Next, at the L4 larval stage, 5 worms were placed in a new NGM plate with *E. coli*. The worms were transferred to fresh plates daily until reproduction was ceased and the total offspring (brood size) *per* worm was estimated. After 24 h the number of hatched eggs was counted, representing the L1 stage animals that were alive in the plates. They were counted as D1, D2 and D3 (Day 1, Day 2 and Day 3 respectively) and the sum from those three days represented the total offspring.

2.4. Behavioral tests

In order to evaluate behavioral parameters related to the cholinergic system, following MeHg, Mn or simultaneous exposure, N2 worms were washed three times and approximately 50 worms per treatment were transferred to NGM plates seeded with *E.coli* OP50 and analyzed 48 h post-exposure. The pharyngeal pumping rate was assessed with a microscope by observing the number of pharyngeal contractions during a 10 second interval. Number of body bends was selected for analysis of locomotion. Worms were transferred to NGM plates without *E. coli* OP50 and the number of body bends was observed with a stereomicroscope over a 20 second interval. Analyses were carried out in at least 5 worms per group, in triplicate. Assays were repeated independently at least four times.

Mutant strains were used in locomotion tests in the presence or absence of bacteria, using the same protocol cited above.

2.5 RNA isolation and real-time polymerase chain reaction (RT-qPCR)

N2 worms were analyzed 0- and 24 h post MeHg, Mn or co-exposure for gene expression related to metal cellular responses. After 24h RNA from at least 20,000 worms per condition was isolated with Trizol followed by chloroform extraction (Chomezynki and Mackey, 1995). One μg of input RNA was reverse transcribed to cDNA by using an Applied Biosystems' high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Expression analysis was performed by Custom TaqMan® Array Analysis using the corresponding TaqMan® Gene Expression Assays for mitochondrial superoxide dismutase/*sod-3* (Ce02404515_g1), superoxide dismutase/*sod-4* (Ce02451138_g1), catalase/*ctl-3* (Ce02406266_g1), vesicular monoamine transporter/*cat-1* (Ce02495612), acetylcholinesterase/*ace-2* (Ce02415460). Target gene expression was normalized to the expression values of *afd-1* (Ce02414573_m1). The relative quantification was determined with the $2^{-\Delta\Delta Ct}$ method and data were expressed as fold change in mRNA levels compared to control worms. Experiments were carried out independently in three worm preparations, each in triplicate.

2.6 Cholinergic degeneration

To assess cholinergic neurodegeneration, we used the LX929 *unc-17::GFP* strain, which has green fluorescent protein (GFP) expressed in all cholinergic neurons. Twenty-four h after exposure to the different doses of Mn, MeHg or both, worms were anaesthetized with 0.02% tetramisole in M9. Fluorescence observations were performed with an epifluorescence microscope (Nikon Eclipse 80i, Nikon Corporation, Tokyo, Japan) equipped with a Lambda LS Xenon lamp (Sutter Instrument Company) and Nikon Plan Fluor 20× dry and Nikon Plan Apo 60 × 1.3 oil objectives. Microscopes were housed in air-conditioned room (20–22°C). Cholinergic neurodegeneration ranged from 0 to 3, where 0 means no degeneration in the cholinergic system in the head, body or tail; 1 means low degeneration in the head, body or tail, 2 means moderate degeneration in the head, body or tail and 3 means high degeneration in the head, body or tail of *C. elegans*. In all experiments the degeneration was assessed by only one observer who was blinded to the treatments. Representative images for the assays were obtained with a PerkinElmer spinning disk confocal, 40 X objective (PerkinElmer, Waltham, MA, USA).

2.7. Mn, Hg and zinc quantification by ICPMS

2.7.1. Standards—For Mn and Zn determination by ICP-MS a calibration curve in the range of 0.025 a 10 μg L-1 was prepared from the dilution of a multielement stock standard solution containing 10 mg L-1 (SCP33MS, SCP Science, Quebec, Canada) in 5% (v/v) HNO3. For Hg determination, the curve (0.5 a 10 μg L-1, prepared in 5% HNO3 and 5% HCl) was obtained from the dilution of a reference solution of 1000 mg L-1 Hg (mercury(II) nitrate in 0.5 mol L-1, Merck, Germany).

2.7.2. Sample preparation procedure—Sample preparation was previously described by Bornhorst et al. (2014). Fifty thousand synchronized L1 worms were acutely treated with MnCl2 or MeHg. Worms were pelleted, washed five times in 85 mM NaCl and re-suspended in 1 mL 85 mM Na l supplemented with 1% protease inhibitor. After sonication, an aliquot was taken for protein quantification using the bicinchoninic acid (BCA) assay-kit (Thermo Scientific). Subsequently, the suspension was mixed again and evaporated and frozen. For the determination of the total concentration of Mn, Zn and Hg, the samples were decomposed with nitric acid. The decomposition was performed in polypropylene flasks were 100 μL of HNO_3 (65%, 1.4 kg L-1, Sigma Aldrich, USA) was added to the sample (mass in the range of 1 to 3 mg) and allowed to stand for 24 h at ambient temperature. Next, 1.25 mL of 5% HCl (37%, 1.19 kg L-1, Merck) was added and left to stand at least 24 h. Analyte determination was performed directly from the solution or diluted accordingly when necessary.

2.7.3. Instrumentation—An inductively coupled plasma mass spectrometer (Perkin Elmer Sciex, Model ELAN DRC II, Thornhill, Canada), equipped with a concentric nebulizer (Meinhard Associates, Golden, USA), a cyclonic spray chamber (Glass Expansion, Inc., West Melbourne, Australia), was used for Mn, Zn and Hg determination. Table 1 describes the conditions for the analyses.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA). The results were plotted as the mean ± SEM (standard error of the mean) of at least four independent experiments. Dose–response lethality curves and LD50 determination were generated using a sigmoidal dose–response model and analyzed with the extra sum-of-squares F test method. One or two-way analysis of variance (ANOVA) followed by the Tukey post hoc test was used to compare the groups. The statistical significance was set at $p < 0.05$.

3 Results

3.1 Manganese (Mn) and mercury (MeHg) exposures reduce the survival of *C. elegans*

The LD_{50} for Mn was 17.78 mM (Fig. 1A), whereas the LD_{50} for MeHg was 30.63 μM (Fig. 1B). Once the LD_{50} was obtained, the remainder of the experiments were carried out at 0, 10, 25 and 50 mM for Mn and 0, 10, 25 and 50 μM for MeHg. When the worms were co-exposed to both metals the groups were referred to as:10+10, 25+25 and 50+50. As shown in Fig. 1C, Mn reduced worm survival at doses above 25 mM [$F_{(3,18)}$=22.03; P<0.001].

MeHg reduced the percent survival only at 50 μM [$F_{(3,27)}$=176.6; P < 0.001]. Co-exposure to 10 mM Mn *plus* 10 μM MeHg further reduced the percent survival in *C. elegans* [$F_{(3,28)}$ = 27.9; P < 0.001]. A similar effect was also observed for co-exposures at higher doses (25+25 and 50+50, P < 0.001). Statistical analysis (two-way ANOVA) showed a significant vehicle or Mn *versus* vehicle or MeHg interaction only for the 50+50 dose [$F_{(1,25)}$=50.65; < 0.0001].

3.2 Behavioral tests

MeHg and Mn alone and in combination induced a delay in worm development initiating from 25 μM to MeHg and from 25 mM to Mn. We determined this by observing worm morphology. Therefore, only worms that reached the L4 stage were analyzed for behaviors to discard potential differences inherent to developmental delays.

3.2.1. Effects of Mn, MeHg and their interactive effect on pharyngeal pumping in *C. elegans*.—Mn and MeHg did not alter pharyngeal pumping in the range of 10 to 50 mM [$F_{(3,18)}$=1.608, P > 0.05; Fig. 2A; $F_{(3,19)}$=1.728, P > 0.05; Fig. 2B]. As shown in Fig. 2C, worms co-exposed to 50 mM Mn *plus* 50 μM MeHg had a significantly lower pharyngeal pumping rate when compared to the control group [$F_{(9,60)}$=2.964; P < 0.05,]. Statistical analysis (two-way ANOVA) showed a significant vehicle or Mn *versus* vehicle or MeHg interaction only for the 25+25 dose [$F_{(1,21)}$=10.58, P < 0.01] and 50+50 dose [$F_{(1,21)}$=13.59, P < 0.01].

3.2.2. Effects of Mn and MeHg on C. elegans locomotion—Mn reduced the number of body bends at doses above10mM [$F_{(3,27)}$=49.46, P < 0.001, Fig. 3A]. MeHg reduced the number of body bends at doses above 10 μM [$F_{(3,20)}$=42.29, P < 0.001, Fig. 3B]. Mn and MeHg alone or in combination also reduced the number of body bends [$F_{(9,63)}$=28,91, < 0.001, Fig. 3C]. Statistical analysis (two-way ANOVA) showed a significant vehicle or Mn *versus* vehicle or MeHg interaction for the 10+10 dose [$F_{(1,20)}$=15.06; P<0.001], 25+25 [$F_{(1,23)}$=32.32, P<0.001] and 50+50 [$F_{(1,25)}$=88.75, P<0.001].

3.3 Mn and MeHg reduce the brood size

The effects of MeHg and Mn on reproduction was assessed by counting the total number of offspring per worm. As shown in Fig. 4, Mn exposure had no effect on brood size at doses of 10 and 50 mM (P > 0.05). Reproduction upon exposure to MeHg at 10 and 50 μM was also statistically indistinguishable from controls (P > 0.05). However, co-exposure to 50 mM Mn *plus* 50 μM MeHg significantly reduced the brood size [$F_{(6,32)}$=2.749, P < 0.05]. Statistical analysis (two-way ANOVA) showed no significant vehicle or Mn *versus* vehicle or MeHg interaction for the 10+10 dose [$F_{(1,15)}$=0.450, > 0.05] and the 50+50 dose [$F_{(1,15)}$=0.325, P > 0.05].

3.4 *Effects of exposure of Mn, MeHg on the gene expression of antioxidant enzymes, SOD-3, SOD-4* and catalase

Mn and MeHg alone did not alter mRNA content for *sod-3* (P > 0.05). In contrast, co-exposure to 50 mM Mn *plus* 50 μM MeHg significantly increased the mRNA content of *sod-3* [$F_{(6,27)}$=8.500, P < 0.001; Fig. 5A]. Statistical analysis (two-way ANOVA) showed a

significant vehicle or Mn *versus* vehicle or MeHg interaction only for the 50+50 dose [$F_{(1,12)}$=6.772, P < 0.05] indicating a synergistic effect between Mn and MeHg on the expression of *sod-3.*

Mn at 50 mM increased mRNA content of *sod-4* (P<0.001). A similar effect was observed for treatments co-exposure to 50 mM Mn *plus* 50 μM MeHg [$F_{(6,25)}$=6,906, P < 0.001; Fig. 5B]. Statistical analysis (two-way ANOVA) showed no significant vehicle or Mn *versus* vehicle or MeHg interaction for the 10+10 dose [$F_{(1,11)}$=1.897, P > 0.05] and 50+50 [$F_{(1,11)}$=0.717, P > 0.05].

Mn and MeHg alone did not alter mRNA content of catalase (P > 0.05). However, co-exposure to 50 mM Mn *plus* 50 μM MeHg led to a significant increase in mRNA content of this enzyme [$F_{(6,29)}$=5.437, < 0.001; CTL, Fig. 5C]. In addition, statistical analysis (two-way ANOVA) showed a significant vehicle or Mn *versus* vehicle or MeHg interaction only for the 50+50 dose [$F_{(1,12)}$=8.083, P < 0.05] suggesting a synergistic effect between Mn and MeHg on the expression of catalase.

3.5 Impact of Mn, MeHg on the expression of key-proteins of the monoaminergic and cholinergic neurotransmission

Mn and MeHg alone did not alter mRNA content of *cat-1* (P > 0.05). However, co-exposure to 50 mM Mn plus 50 μM MeHg increased mRNA content of *cat-1* [$F_{(6,25)}$=4.596, < 0.01, Fig. 6A]. Statistical analysis (two-way ANOVA) also showed a significant vehicle or Mn *versus* vehicle or MeHg interaction only for the 50+50 dose [$F_{(1,12)}$=5.783, P < 0.05], suggesting a synergistic effect between Mn and MeHg on the expression of *cat-1*

In addition, 50 mM Mn increased the mRNA content of *ace-2* (P < 0.001). MeHg did not alter the mRNA content of *ace-2* at doses between 10 and 50 μM (P > 0.05). Co-exposure to 50 mM Mn *plus* 50 μM MeHg led to increased mRNA content of *ace-* [$F_{(6,27)}$=11,70, P < 0.001; Fig. 6B]. Statistical analysis (two-way ANOVA) showed a significant vehicle or Mn *versus* vehicle or MeHg interaction for dose 10+10 [$F_{(1,12)}$=5.998, P < 0.05] suggesting a synergistic effect between Mn and MeHg on the expression of *ace-2.* In addition, no significant interaction was observed in response to co exposure at the 50+50 dose [$F_{(1,12)}$=3.055, P > 0.05].

3.6 Effects of Mn, MeHg on cholinergic degeneration in C. elegans

Mn caused cholinergic neurodegeneration at 25 and 50 mM (P < 0.01). MeHg did not induce cholinergic neurodegeneration at any of the tested doses (20–50 μM, P > 0.05). Co-exposure to both metals induced cholinergic neurodegeneration at all the tested doses [$F_{(9,43)}$=27,83, P < 0.001]. tatistical analysis (two-way ANOVA) showed no significant vehicle or Mn *versus* vehicle or MeHg interaction for all doses: 10+10 [$F_{(1,14)}$ = 0,168, > 0.05], 25+25 [$F_{(1,14)}$ = 2,618, P > 0.05] and 50+50 [$F_{(1,14)}$=0.493, P > 0.05]. These data suggest additive effects of the metals on cholinergic neurodegeneration. Representative images for a worm with no degeneration (Fig 7A and B), low degeneration (Fig. 7C and D), moderate degeneration (Fig. 7E) and high degeneration (Fig. 7F) are shown in Fig. 7.

3.7 Mn and MeHg alter locomotion with mutation-dependent effect in the presence and absence of bacteria

Figure 8 shows the number of body bends upon 10, 25 and 50 mM Mn, 10, 25 and 50 μM MeHg and the combined effect of co-exposure to the two metals. Mn and MeHg and co-exposures did not alter the number of body bends in JD217 (with deficient pharyngeal movement dependent on muscarinic activation) [$F_{(12,38)}$=0.661, P > 0.05], DA453 [$F_{(12,38)}$=0.437, P > 0.05] and GG201 [$F_{(12,38)}$=0.990, P > 0.05] strains. In the PR1152 strain, co-exposure to 50 mM Mn *plus* 50 μM MeHg increased the number of body bends in the presence of bacteria [$F_{(12,38)}$=3.661, P < 0.05; Fig. 8C]. Statistical analysis (two-way ANOVA) showed no significant vehicle or Mn *versus* vehicle or MeHg interaction at the 50+50 dose [$F_{(1,8)}$=0.267, P > 0.05]. In the absence of bacteria, no significant differences were observed between the groups in the PR1152 strain [$F_{(12,38)}$=1.881, P > 0.05; data not shown]. In addition, no significant differences were observed in the RB918 strain [$F_{(12,38)}$=0.593, P > 0.05].

3.8 Mn, MeHg and Zn levels in the C. elegans

A significant increase in Mn levels was observed in the Mn group (exposed to 50 mM) [$F_{(2,6)}$=4.984, P < 0.05; Fig. 9A]. An increase in MeHg level was observed in the groups exposed to MeHg or co-exposed to MeHg with Mn [$F_{(2,6)}$=20.23, P < 0.01, Fig. 9B]. No significant differences were observed in the Zn levels in the different groups when compared to the control group [$F_{(3,8)}$= 0.1866, > 0.05, graph 9C]. Statistical analysis (two-way ANOVA) showed no significant vehicle or Mn *versus* vehicle or MeHg interaction in the 50+50 dose [$F_{(1,8)}$=0.431, P > 0.05, graph 9C].

4 Discussion

Little is known about the health risks associated upon combined exposures to metals, and their interactions at the cellular or molecular levels (Moyson et al., 2017). This is a timely issue to be investigated, as exposure to metal mixtures represents real-life scenarios. The main question addressed herein was whether the simultaneous exposure to MeHg and Mn has greater effects on *C. elegans vs*. single metal exposures? The first result that obtained was the LD_{50} values for Mn and MeHg. TheLD_{50} for Mn was 17.78 mM (Fig. 1A) whereas the LD50 for MeHg was 30.63 μM (Fig. 1B). We selected three doses, one close, one below and one approximating this value to assess the sequalae of co-exposures in the remainder of the experimental protocols. Our experimental design did not allow us to discern what effects are synergistic and what are additive.

Exposure to MeHg and Mn has been shown to increase ROS generation and enhance mitochondrial permeability (Cecatelli et al.,2010; Martinez-Finley et al., 2013; Angeli et al., 2014). Superoxide dismutase (SOD-3 and SOD-4) and catalase (CTL-3) are important enzymes in the regulation of oxidative stress. SOD controls superoxide and hydrogen peroxide and catalase controls hydrogen peroxide levels in the cells. SOD-3 is a mitochondrial enzyme that is dependent on Mn (MnSOD) and it is has been shown to promote longevity in *C. elegans*. SOD-4 is an extracellular enzyme dependent on Cu/ZnSOD (Hunter et al., 2015). CTL-3 is present in the peroxisome or mitochondria with

heme serving as a cofactor. Herein, we observed that the expression of *sod-3*, *sod-4* and *ctl-3* was increased upon co-exposures to the high doses of Mn and MeHg. These results are consistent with the raving-up of defense mechanisms to compensate for excessive oxidative stress generated upon simultaneous increases in both superoxide dismutase and catalase.

As locomotion and feeding are useful endpoints for toxicological studies (Anderson et al., 2001; Wang et al., 2017), these parameters were studied upon MeHg and Mn alone, or co-exposures. It is known that exposure to either MeHg or Mn alone alters locomotion, feeding and reproduction in *C. elegans* (McElwee et al., 2011; Chen et al., 2015; Ijmone et al., 2016). We observed that pharyngeal pumping, the number of body bends and brood size were altered upon combined exposure to these metals at the highest dose and two-way ANOVA corroborated these results.

The behavioral assays described herein are directly or indirectly related to monoaminergic or cholinergic transmission. Specifically, we studied the expression of two important proteins: vesicular monoamine transporter (VMAT/*cat-1*) and acetylcholinesterase (*ace-2*). VMAT is a transporter related with the loading of monoamines into synaptic vesicles and *ace-2* is an enzyme that hydrolyzes ACh into acetate and choline mainly in neuronal cells in *C. elegans*. *ace-2* regulates ACh level, which is the most abundant excitatory neuro transmitter in the nematode. *C elegans* (Rand, 2007). Our results showed that the expression of both proteins was enhanced upon co-exposure to the metals at the highest dose. We suggest that the storage of monoamines and the degradation of ACh may be disrupted, and as a consequence the behaviors related to them are also impaired. Ye & Kim (2015) have previously shown that olfactory Mn uptake enhanced the expression of VMAT in a mouse model, suggesting that this overexpression altered behavioral parameters, mainly related to anxiety. Furthermore, Lohr et al. (2014) showed in a mouse model that increased vesicular monoamine transporter enhanced dopamine release and opposed Parkinson's disease-related neurodegeneration. Thus, disruption in the transport or release of monoamines may promote behavioral changes. Other reports indicate that AChE inhibitors, such as donepezil can up-regulate AChE expression by modulating its gene expression (Kracmarová et al., 2015). However, this does not necessarily imply that the protein's catalytical activity is also increased.

Since we noted alterations in the expression of *ace-2*, next, we decided to evaluate cholinergic neurons morphology for alterations characteristic of degeneration. pproximately 120 neurons in the hermaphrodite *C. elegans* are cholinergic (Rand, 2007), indicating the importance of this system to nematode behavior. Using the *unc-17::GFP* strain we determined that Mn caused cholinergic degeneration at 25 and 50 mM and upon co-exposure to both metals, even at lower doses degeneration was present. It is known that Mn may play an important role in several neurodegenerative disorders such as Parkinson's Disease, Huntington's Disease, Alzheimer's Disease, amyotrophic lateral sclerosis and prion disease (Gunnar et al., 2010; Williams et al., 2010; Roos et al., 2012; Mittereger et al., 2013; Wallin et al., 2016). Additionally, this cholinergic neurodegeneration may explain, in part, the impairment observed in locomotion.

We studied behavioral parameters in selected mutants to assess not only the effects of a binary exposure to metals, but also if the impairments might be related with a specific gene. Co-exposure to 50 mM Mn plus 50 μM MeHg increased the number of body bends causing hyperactivity in *cha-1* mutant worms in the presence of bacteria. Since there is depleted synthesis of ACh in this strain, our data suggest that the hydrolysis of ACh might be impaired, or alternatively the metals might alter other excitatory neurotransmitter systems.

Metal analyses showed that Mn and MeHg accumulated in the worms. Interestingly, the interaction between n (biometal) and MeHg (trace metal) at the higher doses did not increase worm Mn levels, while Hg levels were enhanced. et al. (2014) found that exposure to Mn can alter iron and calcium homeostasis. We also analyzed if Mn or MeHg altered zinc concentrations, as the latter is an important regulator of *sod-4* activity. No difference in worm Zn concentration was observed at any of the exposure paradigms, indicating Zn metabolism is not altered by Mn, MeHg or co-exposure to these metals.

5 Conclusions and perspectives

Combined exposure to Mn and MeHg is more deleterious than exposure to each metal alone. Different behavioural parameters were analysed, corroborating the toxicity of these metals. We also studied the effect of Mn and MeHg on developmental delays in *C. elegans*. Several animals that remained alive were unhealthy in appearance at the high dosing groups (25 and 50 mM). Enzymes associated with the antioxidant system, namely, catalase and superoxide dismutase, were both up-regulated, likely showing an adaptive response to counteract the enhancement in ROS generation. Both, the cholinergic and monoaminergic systems were up-regulated, impairing behavioural parameters related to these neurotransmitters. In conclusion, our studies attest to the need for further inquiry on the effects of metal interactions, since in the environment exposures to mixtures with possible synergetic or additive effects are likely to occur.

Acknowledgments

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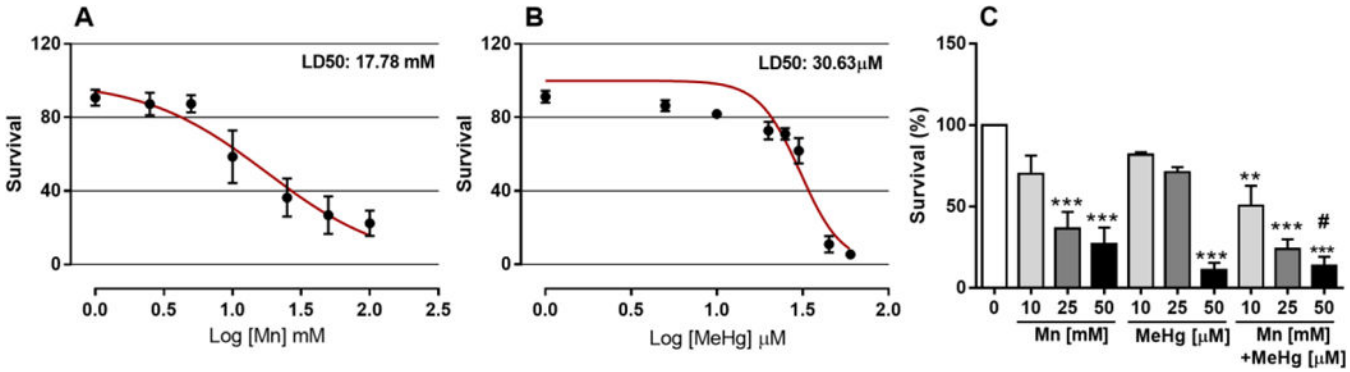


Fig. 1.

Survival percentage of *C. elegans* and lethal dose 50% (LD) for manganese (Fig. A, Mn in mM) and mercury (Fig. B, MeHg in μM) 24h after exposure. The LD_{50} values were determined by non-linear regression analyses with a sigmoid concentration–response equation. Effect of exposure to Mn, MeHg and interaction on the survival of *C. elegans* (Fig. C). Data are expressed as mean ± SEM. $P < 0.05$ was considered to represent a significant difference, n=4 – 8. *Denotes a significant difference from the vehicle group (one-way ANOVA followed by *post hoc* Tukey's, *denotes $^{<}$ 0.05, **denotes P $^{<}$ 0.01 and ***denotes P $^{<}$ 0.001). $^{\#}$Denotes a significant interaction between treatments (two-way ANOVA, $^{\#}$ denote P $^{<}$ 0.05).

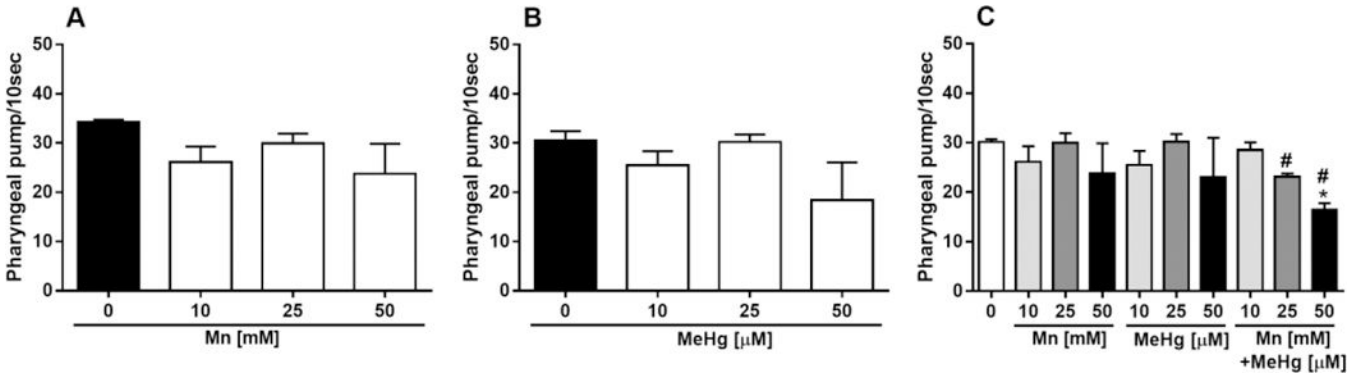


Fig. 2.

Effect of manganese (Fig. A, Mn in mM) and mercury (Fig. B, MeHg in μM) on the number of pharynx pumps *per* 10 seconds of L4-larval stage *C. elegans*. Effect of exposure to Mn, MeHg and interaction on pharynx pumps of *C. elegans* (Fig. C). Data are expressed as mean ± SEM. $P < 0.05$ was considered to represent a significant difference n=4 – 8. *Denotes a significant difference from the vehicle group (one-way ANOVA followed by *post hoc* Tukey's, *denotes $P < 0.05$, **denotes $P < 0.01$ and ***denotes $P < 0.001$). #Denotes a significant interaction between treatments (two-way ANOVA, # denote $P < 0.05$).

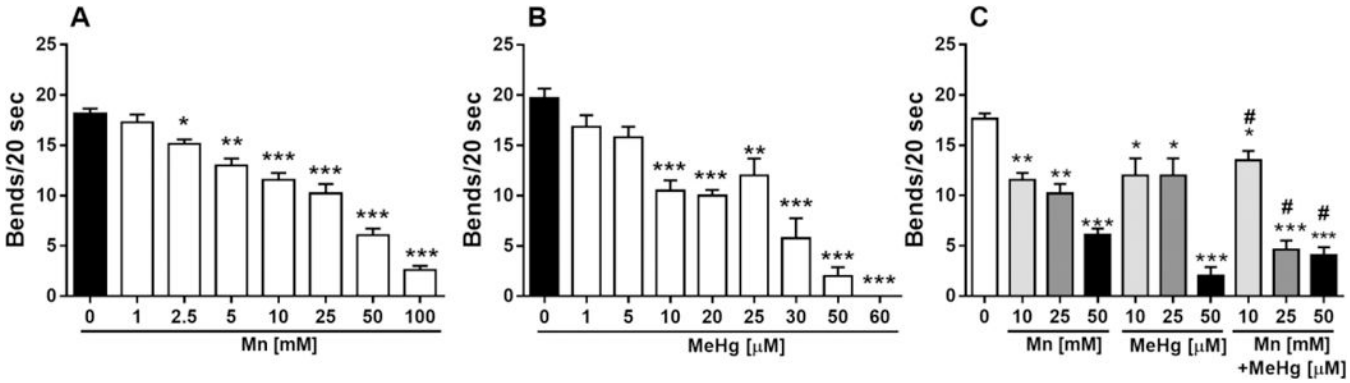


Fig. 3.

Effect of manganese (Fig. A, Mn in mM) and mercury (Fig.B, MeHg in μM) on body bends *per* 20 seconds of L4-larval stage *C. elegans*. Effect of exposure to Mn, MeHg and interaction on body bends of *C. elegans* (Fig. C). Data are expressed as mean ± SEM. P < 0.05 was considered to represent a significant difference, n=4 – 8. *Denotes a significant difference from the vehicle group (one-way ANOVA followed by *post hoc* Tukey's, *denotes P ˂ 0.05, **denotes ˂ 0.01 and ***denotes P ˂ 0.001). #Denotes a significant interaction between treatments (two-way ANOVA, # denote P ˂ 0.05).

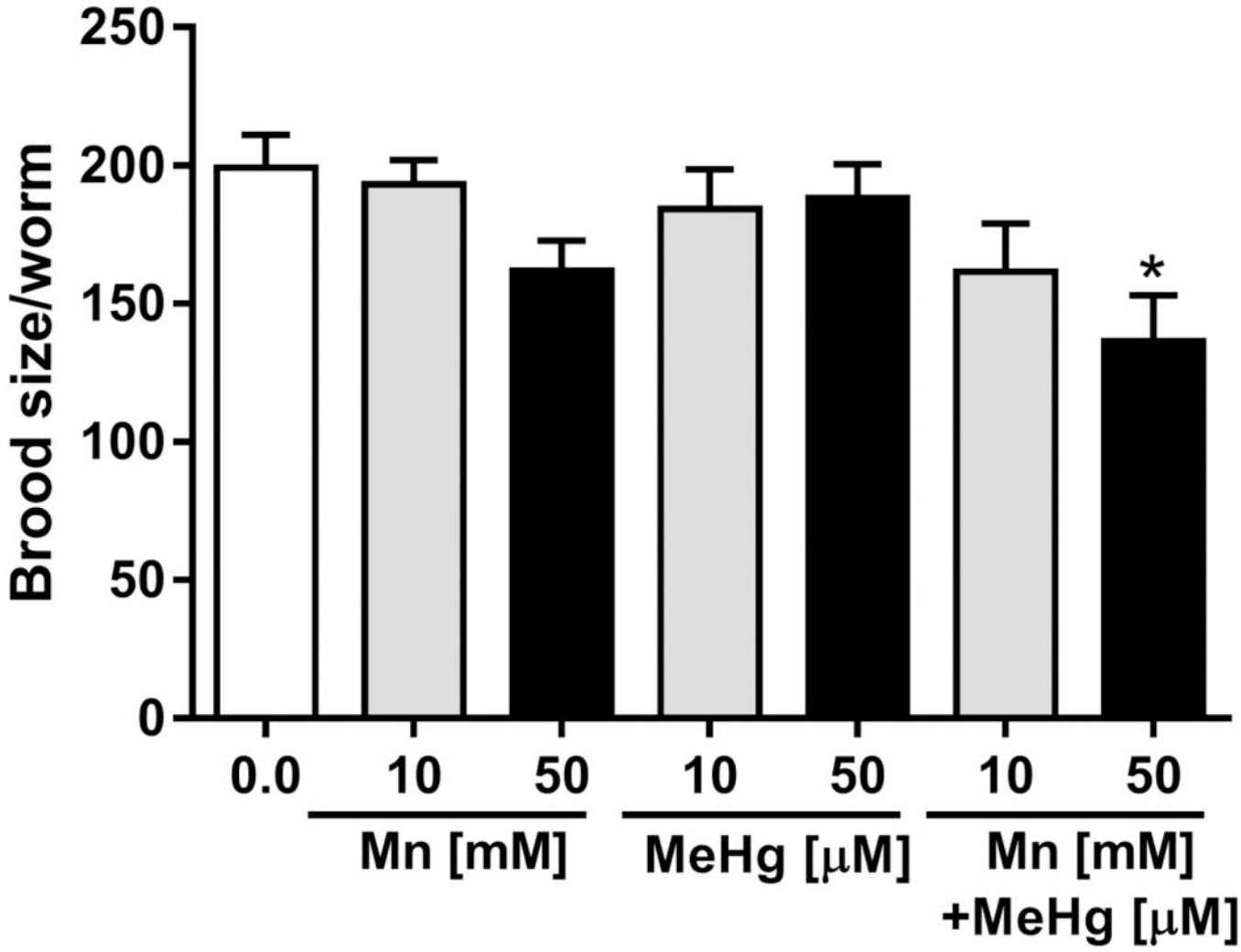


Fig. 4.

Effect of manganese (Mn in mM) and methylmercury (MeHg in μM) and interaction on brood size. Data are expressed as mean ± SEM. $P < 0.05$ was considered to represent a significant difference. *Denotes a significant difference from the vehicle group (one-way ANOVA followed by post hoc Tukey, n=4–5).

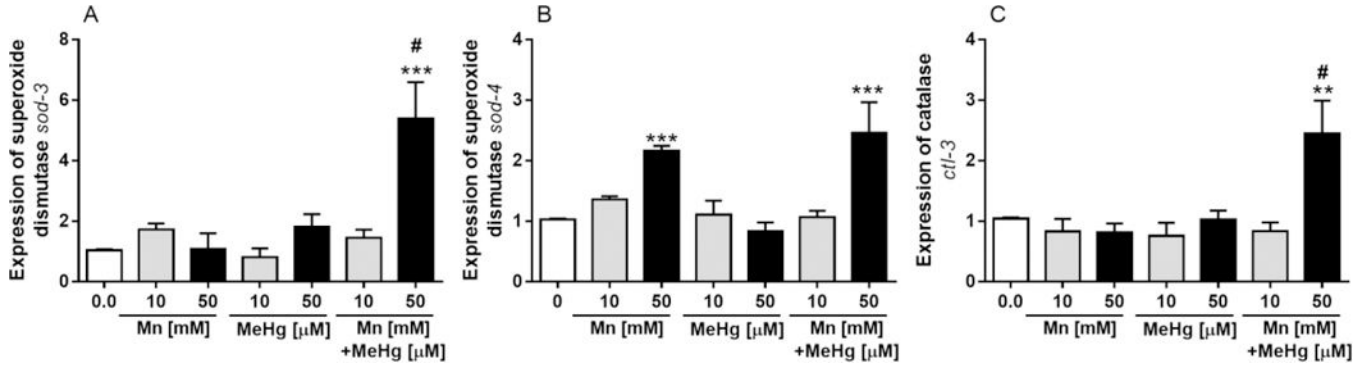


Fig. 5.

Effect of manganese (Mn in mM) and methylmercury (MeHg in μM) on mRNA content of superoxide dismutase 3 (Fig. A, *sod-3*), superoxide dismutase 4 (Fig. B, *sod-4*) and catalase (graph C, *ctl-3*) 24h after exposure. Data are expressed as mean SEM. $P < 0.05$ was considered to represent a significant difference. *Denotes a significant difference from the vehicle group, n=3–4. *Denotes a significant difference from the vehicle group (One-way ANOVA followed by *post hoc* Tukey's, *denotes $P < 0.05$, **denotes $P < 0.01$ and ***denotes $P < 0.001$). #Denotes a significant interaction between treatments (Two-way ANOVA, # denote $P < 0.05$).

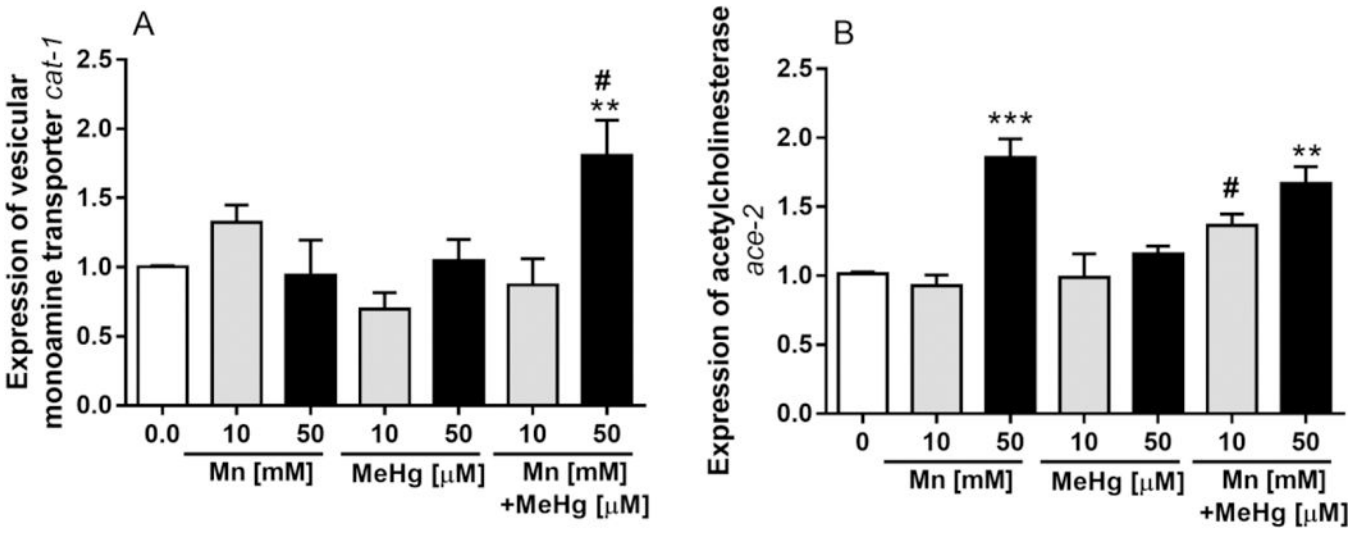
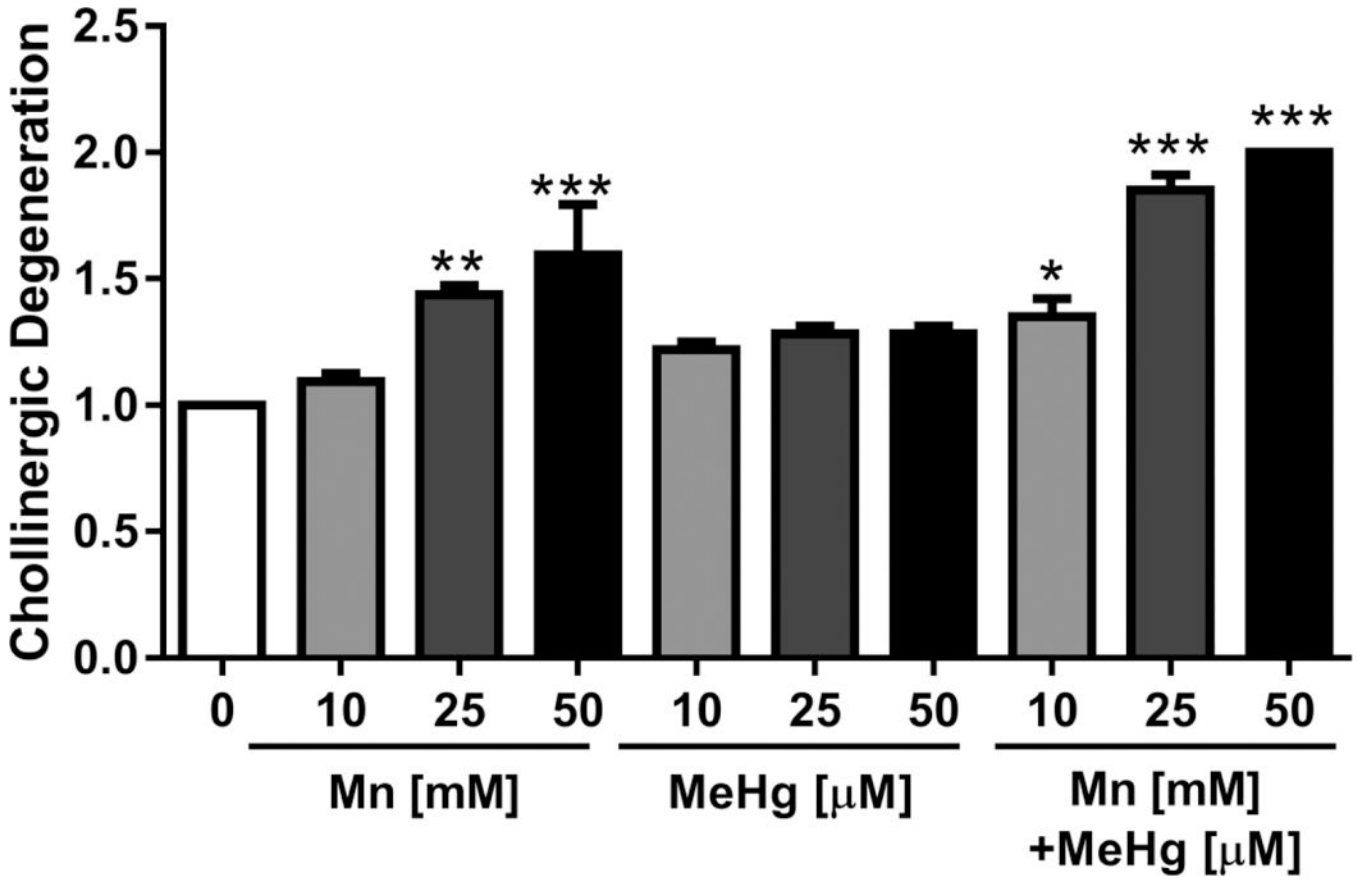


Fig. 6.

Effect of manganese (Mn in mM) and methylmercury (MeHg in μM) on mRNA content of vesicular monoamine transporter (Fig. A, *cat-1*) and acetylcholinesterase (Fig. B, *ace-2*) 24h after exposure. Data are expressed as mean ± SEM. $P < 0.05$ was considered to represent a significant difference, n=3–4. *Denotes a significant difference from the vehicle group (One-way ANOVA followed by *post hoc* Tukey's, *denotes $P < 0.05$, **denotes $P < 0.01$ and ***denotes $P < 0.001$). #Denotes a significant interaction between treatments (Two-way ANOVA, # denote $P < 0.05$).



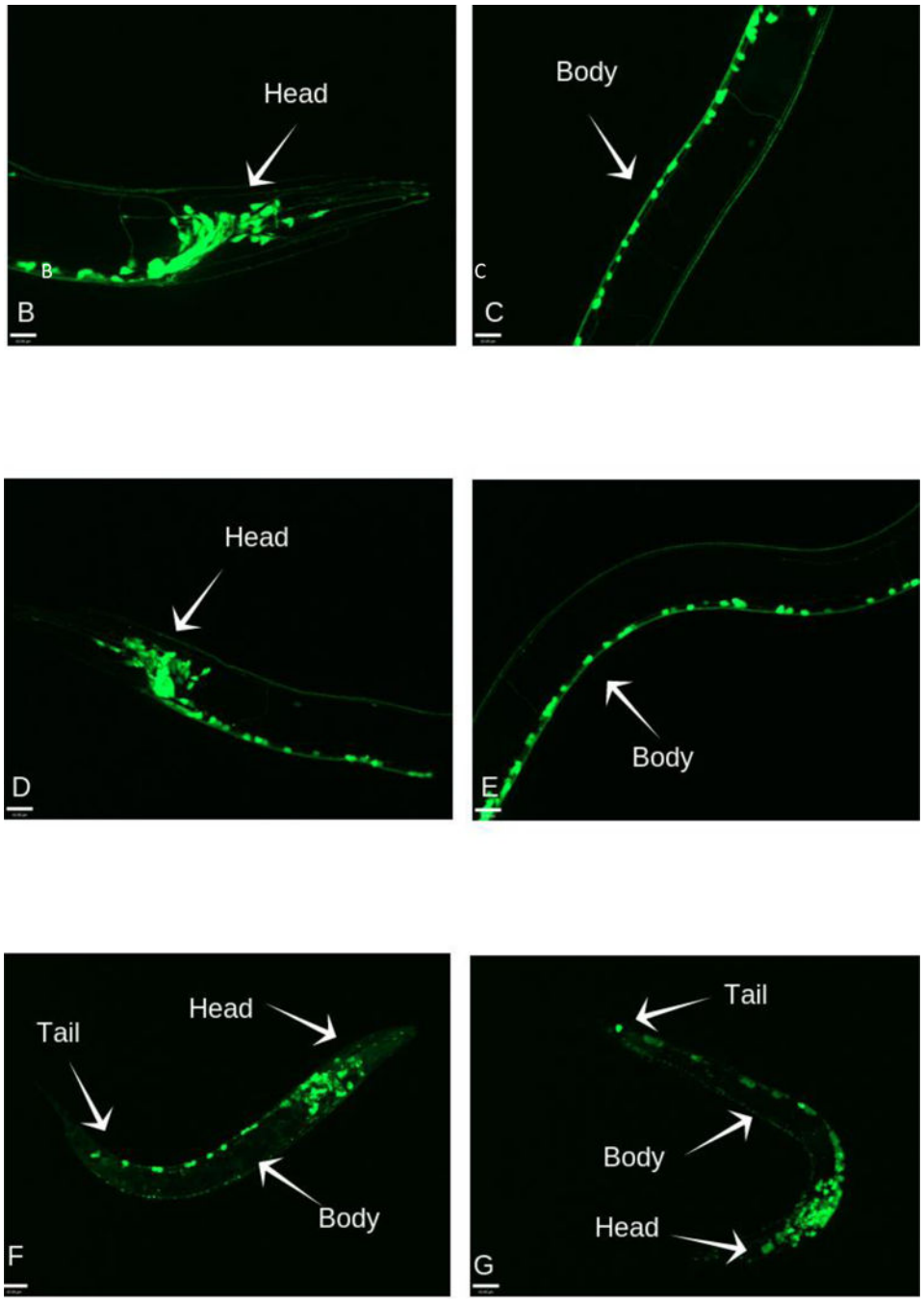


Fig. 7.

Representative images of the effect of manganese (Mn in mM) and methylmercury (MeHg in μM) on cholinergic degeneration in C*. elegans* 24h after exposure. Fig. 7B and C represent the grade 0 of degeneration where no degeneration in the cholinergic system was found in the head, body or tail (grade 0); Figures 7D and E are representative from low degeneration in the head, body or tail (grade 1), Figure 7F is representative from moderate degeneration in the head, body or tail (grade 2) and Figure 7G is representative from high degeneration in the head, body or tail (grade 3) of *C. elegans*. Data are expressed as mean ±

SEM. $P < 0.05$ was considered to represent a significant difference, n=4 – 8. *Denotes a significant difference from the vehicle group (One-way ANOVA followed by *post hoc* Tukey's, *denotes $P < 0.05$, **denotes < 0.01 and ***denotes $P < 0.001$).

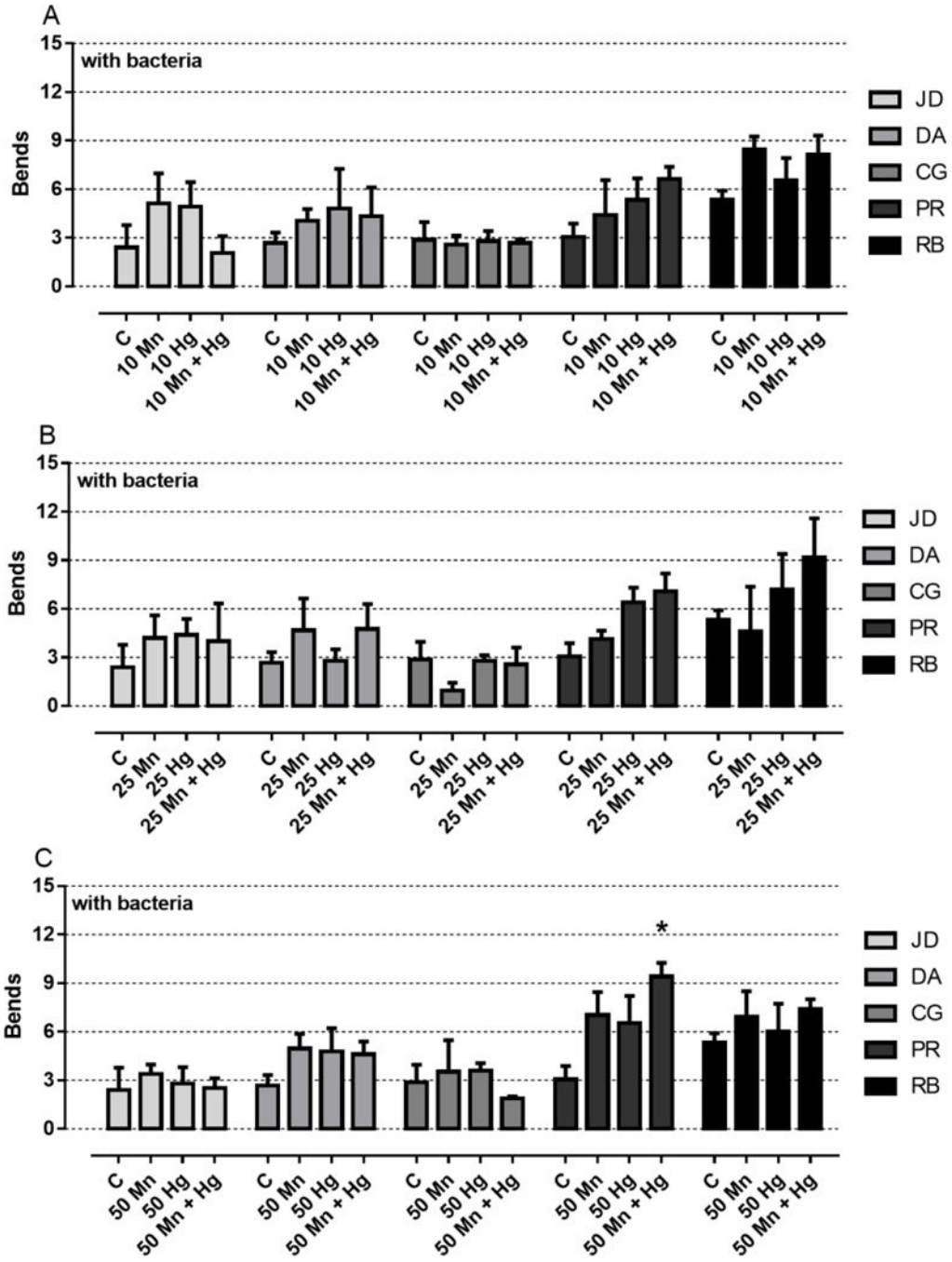


Fig. 8.

Effects of manganese (Mn in mM), methylmercury (MeHg in μM) and interaction on the number of body bends for 20s in different strains of *C. elegan* at L4-larval stage: DA453 (*eat-2*), RB918 (*acr-16*), JD217 (gar-3), GG201 (*ace-1/ace-2*) and PR1152 (*cha-1*) in the presence of bacteria. Data are expressed as mean ± SEM. $P < 0.05$ was considered to represent a significant difference, n=3. *Denotes a significant difference from the vehicle group (One-way ANOVA followed by *post hoc* Tukey's, *denotes $P < 0.05$, **denotes $P < 0.01$ and ***denotes $P < 0.001$).

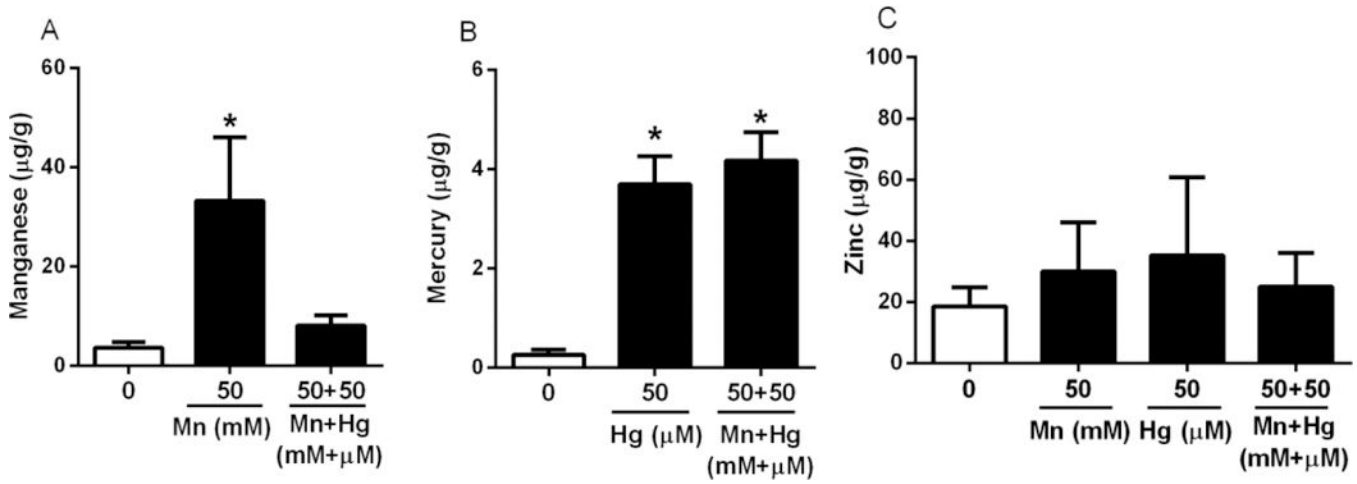


Fig. 9.

Manganese (Mn in mM, Fig. A), methylmercury (Hg in μM, Fig. B) and Zinc (Zn, Fig. C) levels (in μg/g) in the *C. elegans* 24h after exposure to manganese and mercury. Data are mean ± SEM. $P<0.05$ was considered to represent a significant difference. *Denotes a significant difference from the vehicle group (One-Way ANOVA test followed by post hoc Holm-Sidak's multiple comparisons test, n=3).

Table 1.

Conditions for measurements

Parameter	ICP-MS
RF power, W	1300
Plasma gasflow rate, L min^{-1}	15
Auxiliary gas flow rate, L min^{-1}	1,2
Nebulizer gas flow rate, L min^{-1}	1,00
Dweell time, ms	40
Reading	5
Readings per replicate	3
Isotope, m/z	^{202}Hg, ^{55}Mn, ^{66}Zn