

# Review of the Ecotoxicological Properties of the Methylenedianiline Substances

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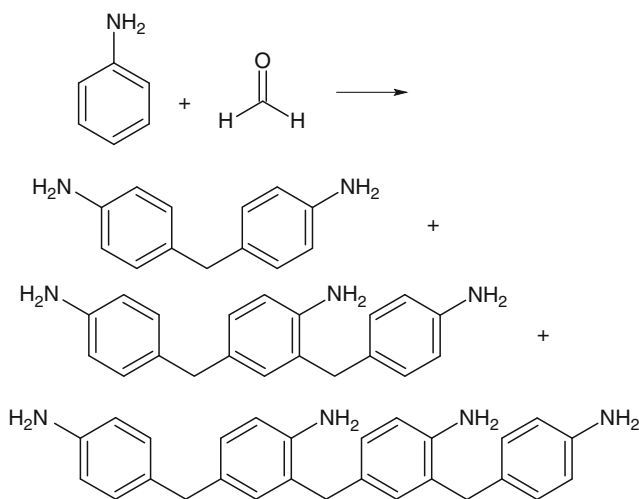
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## 1 Introduction

The methylenedianiline substances (MDAS) are a family of high production volume chemicals with annual world production volume estimated to exceed four million metric tons per year (Carvajal-Diaz 2015). More than 98 % of this production is consumed as an intermediate for the production of the methylenediphenyl diisocyanate (MDI) substances, which are important monomers for the versatile thermoset polymer group of polyurethanes. Minor amounts of the MDAS are also consumed in manufacture of high performance polyimide fibers, and in manufacture of other specialty chemicals and resins.

The industrial-scale production of the MDAS occurs via a condensation reaction of aniline and formaldehyde, as shown in Fig. 1.

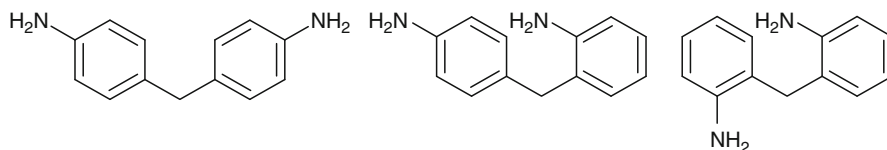
The bulk product of this reaction is commonly referred to as polymeric methylenedianiline (pMDA), and the relative proportions of the illustrated components can be controlled by adjusting the ratio of aniline and formaldehyde reactants. It should be noted here that the name “polymeric MDA” does not necessarily indicate that this reaction mixture meets the current OECD definition of “polymer”. In many instances, due to > 50 % of composition coming from 4,4'-methylenedianiline, the “polymeric MDA” reaction product would not meet this OECD definition (<http://www.oecd.org/env/ehs/oecddefinitionofpolymer.htm>). The pMDA reaction products can be further isolated or purified by fractional distillation, making possible any number and combination of the substances listed in Table 1 (Alport et al. 2003). The 2:1 condensation products are sometimes called “2-ring-MDA” but are hereafter referred to as methylenedianiline (MDA). When isolated from the bulk pMDA reaction mixture the 2-ring MDA will typically occur



**Fig. 1** Generalized commercial synthesis route of the methylenedianiline substances (MDAS)

**Table 1** Identity of the commercially-relevant methylenedianiline substances (MDAS)

Substance	Chemical name	Chemical Abstracts Registry Number	Synonyms	Typical % in MDA	Typical % in pMDA
Polymeric Methylene-dianiline (pMDA)	Formaldehyde, polymer with benzenamine	25214-70-4	pMDA	N/A	100
Methylenedianilines (MDA)	Benzenamine, 4,4'-methylenebis-	101-77-9	4,4'-MDA	90–95	50
	Benzenamine, 2, 4'-methylenebis-	1208-52-2	2,4'-MDA	2–5	
	Benzenamine, 2,2'-methylenebis-	6582-52-1	2,2'-MDA	<1	
Oligomeric methylenedianilines	3-ring	N/A	oMDA	N/A	25
	4-ring			N/A	12
	5-ring			N/A	6
	Higher oligomers			N/A	<6

**Fig. 2** Molecular structures of the 4,4'-; 2,4'-; and 2,2'-isomers of methylenedianiline

as a mixture of three positional isomers (Fig. 2), where the 4,4'-MDA is the predominant isomer representing more than 90–95 % of the isomer mixture, with the 2,4'-MDA and 2,2'-MDA making up 2–5 % and less than 1 % of the mixture, respectively. The higher oligomer components of pMDA are sometimes named “3-ring-MDA”, “4-ring-MDA” and so on, and are hereafter referred to as oligomeric MDA or oMDA.

In the evaluation of physical-chemical and toxicological properties of chemical substances, it is desirable to conduct testing on commercially-relevant substances which occur at a high-purity or as a single-component. For this reason, and because it is the most commercially prominent of the methylenedianiline substances, the 4,4'-MDA substance has been intensely investigated for its physical-chemical and toxicological properties. While some properties have been studied for representative pMDA mixtures, very few studies have been conducted on the less prominent isomers of MDA and apparently none on the isolated oMDA homologues. Thus, properties of these latter substances are often inferred (i.e., by read-across) from those of 4,4'-MDA. Though mammalian toxicological hazards are not a subject of this review, it should be mentioned that 4,4'-MDA is classified by several

authorities as a genotoxic carcinogen. In 2001, the European Union issued the EU risk assessment report on 4,4'-MDA which provided a summary of all physical-chemical and hazard property data known by then (European Union 2001). Since that time, and in preparation for the registration under commission regulation 1907/2006 in the EU, some more data on 4,4'-MDA were generated. Robust summaries of these past and more recent studies are now available to the public via the European Chemicals Agency (ECHA) web site (<http://echa.europa.eu/web/guest/information-on-chemicals/registered-substances>). In addition, the government of Canada has recently completed a draft screening level risk assessment for a grouping of the MDA, pMDA, and MDI substances (Government of Canada 2014). These and other regulatory assessments can provide good overviews of the available physical-chemical, health, and environmental properties of the MDAS family, as well as their assessed potential exposures and risks. This current review provides a deeper focus and summary of the known ecotoxicological properties of the MDAS family as available from published and private company studies available through year 2014. A similar in-depth review and summary of environmental fate properties for the MDAS family, including the aspect of bioaccumulation, will be reviewed in a subsequent review article of this journal.

The majority of studies cited in this review and in the aforementioned regulatory assessments of the MDAS have been commissioned by the International Isocyanates Institute, Inc. (III; <http://diisocyanates.org/>) and its private company members, and have not been published previously. For such cited references which are not from publically available sources, the ECHA web site (ECHA 2014a, b) provides robust study summaries for these studies.

## 2 Physical-Chemical Properties

The knowledge of the physical-chemical properties of the MDAS and their environmental behavior is essential to the complete understanding of the ecotoxicological effects of this substance family. The physical-chemical properties of 4,4'-MDA and pMDA are summarized in Table 2. These data for 4,4'-MDA are summarized in the EU risk assessment report (European Union 2001; McNabb *et al.* 1999) and in the more recent robust study summaries provided by ECHA (2014a). The substance tested consisted of 97.39 % 4,4'-MDA, 1.98 % 2,4'-MDA, 50 ppm 2,2'-MDA, and less than 10 ppm aniline.

Physical-chemical properties for pMDA are not that uniform and depend on the composition, as pMDA is an oligomeric mixture which varies slightly in composition based on the ratio of its reactants (Table 1). Robust study summaries of these pMDA properties are also provided by ECHA (2014b).

As weak bases, the water solubilities of the MDAS depend on the pH. Though the individual molecules possess two or more primary amino-groups, their pKa

**Table 2** Summary of relevant physical-chemical properties of the 4,4'-methylenedianiline (MDA) and polymeric methylenedianiline (pMDA) substances

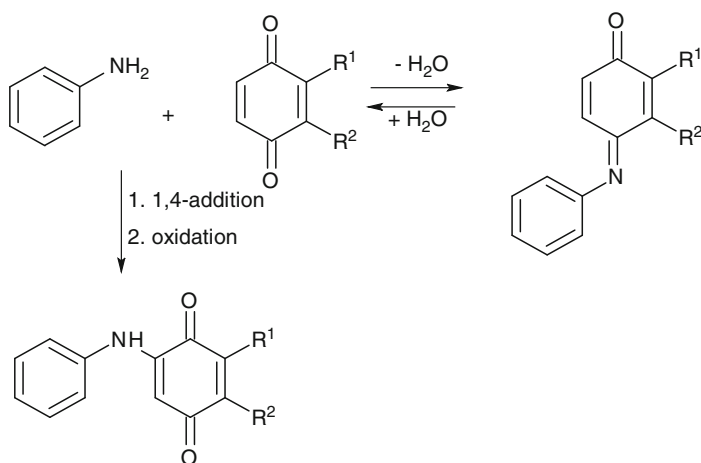
Property	4,4'-Methylenedianiline (MDA)	References	Polymeric Methylenedianiline (pMDA) <sup>a</sup>	References
Physical state at 20 °C	White solid	ECHA (2014a)	Viscous liquid	ECHA (2014b)
Melting point (°C)	90–92	ECHA (2014a)	Glass transition at 0.8 and –2.7	ECHA (2014b)
Boiling point (°C)	398 ± 5 @ 101.3 kPa	ECHA (2014a)	410.6 @ 101.3 kPa	ECHA (2014b)
Bulk density (g/cm <sup>3</sup> , 20 °C)	1.150	ECHA (2014a)	1.150	ECHA (2014b)
Vapor pressure (Pascal)	2.5 × 10 <sup>−4</sup> (25 °C)	ECHA (2014a)	<1 × 10 <sup>−4</sup> (20 °C) 1.6 × 10 <sup>−4</sup> (50 °C)	ECHA (2014b)
Water solubility (g/L at 25 °C)	pH 5.3 = 2.2 pH 7 = 1.01 pH 9 = 0.84	ECHA (2014a)	pH 7 = 0.36–1.22	ECHA (2014b)
Octanol-water partition coefficient (Log Pow)	1.55	ECHA (2014a)	1.2–2.7	ECHA (2014b)
Dissociation constant (pKa at 20 °C)	4.96	ECHA (2014a)	Not determined	N/A

<sup>a</sup>pMDA sample containing 58 % 2-ring-, 23 % 3- ring-, 10 % 4-ring- and 3 % 5-ring-isomers; 6 % higher oligomers

values are essentially indistinguishable by the spectrophotometric method of OECD Guideline 112 (OECD 1981). The apparent equivalence of the pKa values for these amino groups is owed to the fact that delocalization of the positive charge of the corresponding ammonium ions across the molecule is restricted by the methylene group which bridges the aromatic rings.

### 3 Environmental Behavior

The environmental fate behaviors of the MDAS are complex, and while summarized in a separate review, is it relevant to mention here the aspects which influence stability and bio-availability associated with ecotoxicological study. Briefly, like other primary aromatic amines, the MDAS react with natural organic matter in surface waters, sediment, and soil, forming covalent nitrogen-carbon bonds. The 1,4-addition of primary aromatic amines to  $\alpha,\beta$ -unsaturated carbonyl compounds such as quinones is known to serve as a sink for sequestration of these amines in the environment (Parris 1980; Weber et al. 1996; Colón et al. 2002). A generalization of this reaction is illustrated in Fig. 3. Ferulic acid, a derivative of cinnamic acid which for example represents about 1 % wt of cereal plant dry mass, is another



**Fig. 3** Representative reaction of primary aromatic amines with quinones in humic matter, where  $\text{R}^1$  and  $\text{R}^2$  indicate substituent groups of the macromolecular organic matter

reaction partner for covalent binding of primary aromatic amines in the environment, as demonstrated by Tatsumi et al. (1994).

In soil degradation tests, 4,4'-MDA showed an organic carbon-normalized adsorption coefficient ( $K_{\text{OC}}$ ) of 3800 and 5680 in anaerobic and aerobic soils, respectively, after an equilibration time of 8 h (Cowen et al. 1998). The 8 h value achieved approximately 90 % of the value measured after 7 days equilibration time. After 7 days, part of the MDA could be desorbed when equilibrated with fresh 0.1 M  $\text{CaCl}_2$  for 24 h. The high affinity of MDA to soil is most likely attributable to reversible and irreversible binding of the aromatic amino group to organic matter (Tatsumi et al. 1994; Parris 1980; Weber et al. 1996; Li et al. 2000; Colón et al. 2002).

From these examples of primary aromatic amine reactivity in environmental media, it is obvious that the availability of free MDAS has to be checked in ecotoxicological experiments to be able to draw correct and robust conclusions associated with their exposure with organisms. Vice versa, for environmental risk assessment, the potential biological (non)-availability of MDAS in eco-systems needs to be considered when data from controlled, somewhat artificial laboratory experiments are taken as benchmarks.

For the performance of ecotoxicological tests, MDAS usually need to be dissolved in solvents. Although reactions may be possible, for example, Schiff base formation, MDA was shown to be sufficiently stable in acetone, ethyl acetate, acetonitrile and diluted phosphoric acid (Cowen et al. 1996).

## 4 Ecotoxicological Properties

The ecotoxicological properties of MDA will be summarized in the following chapters, with each chapter allocated to the aquatic, benthic, and terrestrial environmental compartments and their associated standardized tests/species.

### 4.1 Toxicity to Aquatic Organisms

In the following, the aquatic taxa bacteria, algae, daphnia and fish will be addressed. Data are summarized in Table 3.

#### 4.1.1 Tests with Bacteria

Fujiwara (1981) investigated the effect of MDA on *Escherichia coli* at 25 °C. In physiological saline, 100 mg/L MDA caused a measurable reduction in cell count over 5 days, whereas 50 mg/L was the no-observed-effect concentration (NOEC). In nutrient broth, 100 mg/L MDA did not cause a reduction in cell count compared to the control.

Caspers et al. (1986) investigated the inhibition of activated sludge by 4,4'-MDA. The composition of the test substance was 99.7 % 4,4'-MDA, 0.25 % 2,4'-MDA and 0.05 % 2,2'-MDA. The test was performed according to guideline OECD No. 209 (1984). After a 3 h contact time, oxygen consumption was measured for 0.5 h. Test concentrations were 1, 10 and 100 mg/L (nominal). At each concentration tested, the inhibition was 15 %. The MDA concentration was below the limit of solubility, and the lack of a dose–response is somewhat odd, but the validity of the test system was checked with the positive control 3,5-Dichlorophenol. The authors concluded the 3 h-EC<sub>50</sub> > 100 mg/L. It should however be noted that EC<sub>50</sub> values for bacteria are dependent on the biomass loading of the tested inoculum. Evidence for inhibition of bacterial respiration has been observed in biodegradation screening tests employing as little as 10 mg/L 4,4'-MDA and 30 mg/L (dry solids) activated sludge (unpublished data of The Dow Chemical Company).

Bringmann and Meinck (1964) reported that MDA inhibits the digestion of glucose by *Pseudomonas putida* at a concentration of about 15 mg/L. The digestion of glucose resulted in a decrease of the pH value from pH = 7.5 to pH = 6.0. The lowest test concentration, which resulted in a higher pH value at the end of the test compared to the control, was called inhibition concentration. Details on the composition of the test substance are not provided.

Kaiser and Palabrica (1991) investigated the effect of 4,4'-MDA (99 % pure) on the luminescent bacterium *Photobacterium phosphoreum* (i.e., *Vibrio fischeri*). The

**Table 3** Summary of 4,4'-methylenedianiline (MDA) toxicity to aquatic organisms

Species	Endpoint and duration	Result (mg/L)	References
Bacteria <i>Escherichia coli</i>	5 days, cell count	LOEC = 100 NOEC = 50	Fujiwara (1981)
Bacteria activated sludge	3 h, respiration inhibition,	EC <sub>15</sub> = 1 EC <sub>50</sub> > 100	Caspers et al. (1986)
Bacteria <i>Pseudomonas putida</i>	16 h, growth	EC <sub>20</sub> = 15 <sup>a</sup>	Bringmann and Meinck (1964)
Marine bacteria <i>Vibrio fischeri</i>	0.5 h, luminescence	EC <sub>50</sub> = 6.6	Kaiser and Palabrica (1991)
Bacteria <i>Ochrobactrum anthropi</i>	35 days, respiration inhibition	EC <sub>50</sub> = 53	Kim et al. (2002)
Fungus <i>Aspergillus</i> sp.	35 days, respiration inhibition	EC <sub>50</sub> = 53	Kim et al. (2002)
Green algae <i>P. subcapitata</i>	72 h	E <sub>b</sub> C <sub>50</sub> = 5.34 E <sub>r</sub> C <sub>50</sub> = 14.4 NOEC <sub>b</sub> = 0.93 NOEC <sub>r</sub> = 9.3	Mitsubishi Chemical Safety Institute (2008a)
Green algae <i>S. subspicatus</i>	96 h; growth	EC <sub>20</sub> = 31 <sup>a</sup>	Bringmann and Meinck (1964)
Green algae <i>S. subspicatus</i>	72 h; E <sub>r</sub> C <sub>50</sub>	21	Rufli and Mueller (1985a)
Green algae <i>S. subspicatus</i>	72 h;	E <sub>b</sub> C <sub>50</sub> = 9.8 E <sub>r</sub> C <sub>50</sub> = 11.0 E <sub>b</sub> C <sub>10</sub> = 2.4 E <sub>r</sub> C <sub>10</sub> = 0.3	European Union (2001)
Green algae <i>C. pyrenoidosa</i>	N.L. <sup>b</sup>	1–10	Rhône-Poulenc Chimie (1977)
Cyanobacterium <i>S. cedrorum</i>	N.L. <sup>b</sup>	1–10	Rhône-Poulenc Chimie (1977)
Marine Diatom <i>N. fustulum</i>	N.L. <sup>b</sup>	10–100	Rhône-Poulenc Chimie (1977)
Crustacean <i>Daphnia magna</i>	EC <sub>50</sub>	5.7 (24 h)	Rufli and Mueller (1985c)
Marine Crustacean <i>Moina macrocopa</i>	EC <sub>50</sub>	2.3 (24 h)	Fujiwara (1982)
Marine Crustacean <i>Moina macrocopa</i>	14-days	0.15	Fujiwara (1982)
Crustacean <i>Daphnia magna</i>	EC <sub>50</sub>	0.25 (48 h)	Bringmann and Meinck (1964)
Crustacean <i>Daphnia magna</i>	EC <sub>50</sub>	8.08 (24 h); 2.47 (48 h)	Mitsubishi Chemical Safety Institute (2008b)
Crustacean <i>Daphnia magna</i>	EC <sub>50</sub>	0.19–0.6 (24 h); 0.019–0.06 (48 h)	Mitsubishi Chemical Safety Institute (2008c)
Crustacean <i>Daphnia magna</i>	21-day	NOEC = 0.00525 LOEC = 0.0182	Mitsubishi Chemical Safety Institute (2008c)

(continued)



**Table 3** (continued)

Species	Endpoint and duration	Result (mg/L)	References
Crustacean <i>Daphnia magna</i>	EC <sub>50</sub>	1.5 (24 h); 0.35 (48 h)	Salinas (2011)
Crustacean <i>Daphnia magna</i>	EC <sub>50</sub>	2.19 (24 h); 0.46 (48 h)	Salinas (2012) <sup>c</sup>
Mollusca <i>Limnea stagnalis</i>	20-h LC <sub>50</sub>	210	Rhône-Poulenc Chimie (1977)
Zebrafish <i>Danio rerio</i>	96-h LC <sub>50</sub>	42	Rufli and Mueller (1985b)
Rainbow trout <i>Oncorhynchus mykiss</i>	96-h LC <sub>50</sub>	39	Rufli and Mueller (1985c)
Zebrafish <i>Danio rerio</i>	96-h LC <sub>50</sub>	65	Caspers et al. (1986)
Golden orfe <i>Leuciscus idus</i>	96-h LC <sub>50</sub>	53	Munk and Kirsch (1988)
Medaka <i>Oryzias latipes</i>	96-h LC <sub>50</sub>	20.6	Mitsubishi Chemical Safety Institute (2008d)

<sup>a</sup>Assumed to be an EC<sub>20</sub> (see text)<sup>b</sup>Noxious limit (see text)<sup>c</sup>56.9 % 4,4'-MDA, 7.1 % 2,4'-MDA, 26 % 3-ring MDA, 10 % 4-ring MDA and higher oligomers

EC<sub>50</sub>, determined as the concentration resulting in 50 % reduction in light emission compared to control after 30 min contact time, was 6.6 mg/L.

In the scope of biodegradation tests with 4,4'-MDA, Kim et al. (2002) demonstrated that at a concentration of 30 mg/L MDA, CO<sub>2</sub> evolution reached about 70–80 % of the theoretical yield over 35 days with non-adapted activated sludge as well as with enrichment cultures of *Ochrobactrum anthropi* or *Aspergillus* sp. These enrichment cultures were generated by the incubation of soil extracts taken from areas where dehydrated activated sludge of the waste water treatment plant was buried with MDA as the sole carbon source. However, at concentrations of 50, 100 or 300 mg/L MDA, CO<sub>2</sub> evolution reached levels of about 50, 20 and 5 % after 35 days, respectively. Data were presented in diagrams and, therefore, any further interpretation has to be regarded with care. If it is assumed that 30 mg/L MDA had no effect on the microorganisms, dosages of 50, 100 and 300 mg/L reduced the CO<sub>2</sub> evolution down to levels of 71, 21 and 7 % for *O. anthropi* and to 57, 14 and 7 % for *Aspergillus* sp. A rough logit analysis of the graphical data given in the paper results in EC<sub>50</sub> values of about 53 mg/L for *O. anthropi* and *Aspergillus* sp., however the concentration of biomass associated with these tests was not quantitatively determined. From the dose–response curves it is not clear whether or not 50 mg/L represent already an inhibition of the microorganisms or whether there is simply a substrate overload; 100 mg/L, however, is clearly inhibiting for *Aspergillus* sp., and for *O. anthropi* the formation of CO<sub>2</sub> comes to a halt after

25 days. Further, at 30 mg/L the CO<sub>2</sub> production rate is virtually the same between the non-adapted sludge and the enrichment cultures; this result indicates toxicity of MDA to MDA-digesting microorganisms.

#### 4.1.2 Tests with Algae

The toxicity of 4,4'-MDA to a freshwater single-celled algae (*Pseudokirchneriella subcapitata*; previously *Selenastrum capricornutum*) was investigated by the Mitsubishi Chemical Safety Institute in 2002, and an English translation was made available as International Isocyanate Institute report 11545 (Mitsubishi Chemical Safety Institute 2008a). The test was performed according to OECD Guideline No. 201 (2006). The purity of the test substance was 99.6 %, and test concentrations were checked by HPLC-UV. The test substance concentration was constant ( $\pm 20$  %) over the exposure time. In the European Union, the median growth rate inhibition concentration ( $E_rC_{50}$ ) is the preferred endpoint; in this article, the endpoint of median biomass inhibition concentration ( $E_bC_{50}$ ) is provided additionally. Against biomass, after 72 h the  $E_bC_{50}$  was 5.34 mg/L (95 % C.I. 3.57–7.98 mg/L) and the  $NOEC_b$  was 0.93 mg/L. In terms growth rate, the  $E_rC_{50}$  was 14.4 mg/L (24–72 h) and the  $NOEC_r$  was 9.3 mg/L.

In a study with the colonial freshwater alga, *Scenedesmus subspicatus*, the 72 h  $E_rC_{50}$  was 21 mg/L (95 % C.I. 11–29 mg/L) for 4,4'-MDA (Rufli and Mueller 1985a). The test was performed according to OECD Guideline 201 (1981). The test substance was named TK 10504 (commercial grade) and claimed to be 4,4'-MDA. Data on purity are not provided. However, the European Union cites this report (2001) and mentions a purity of 95.5–98 %. Results were based on measured concentrations and the data were provided; the analytical method is not mentioned.

Caspers and Mueller (1992) investigated the toxicity of pMDA on *Scenedesmus subspicatus*. After 72 h exposures, the  $E_bC_{10}$  and  $E_bC_{50}$  values were 2.4 and 9.8 mg/L, and the  $E_rC_{10}$  and  $E_rC_{50}$  values were 0.3 and 11.0 mg/L, respectively.

Bringmann and Meinck (1964) reported a limit concentration of 31 mg/L for the inhibition of growth for *Scenedesmus* for 4 days exposure. It is assumed that this level is equal to an  $EC_{20}$ .

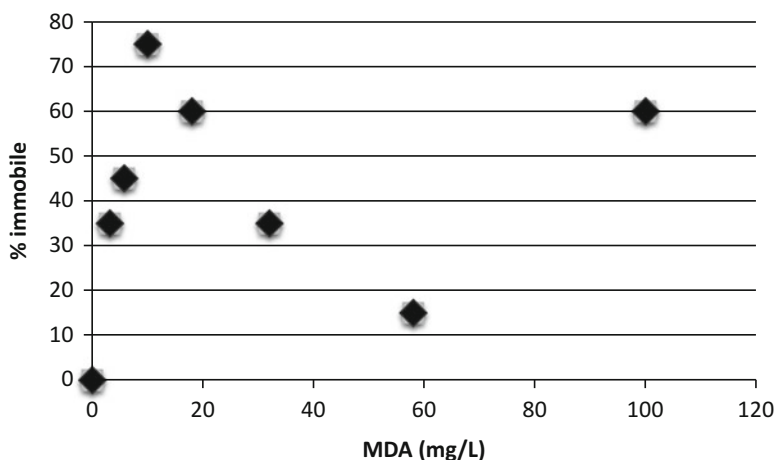
A pMDA sample containing 60 % wt 4,4'-MDA was tested against various algae/cyanobacteria (Rhône-Poulenc Chimie 1977). In those tests, a “noxious limit” was established, which was a concentration where the growth of the organisms was inhibited against a control. Tests were stated to be “run for a time sufficient to detect multiplication of organisms in the control”. For the green alga *Chlorella pyrenoidosa* and the blue-green alga *Synechocystis cedrorum* (i.e., *Cyanobacterium cedrorum*) the noxious limit was in the range from 1.0 to 10.0 mg/L. For the marine diatom *Nitzschia frustulum* the noxious limit fell between 10.0 and 100.0 mg/L. Test concentrations were verified to be controlled within initial nominal values using a photometric method.

As the Mitsubishi studies are the only ones conducted and documented under Good Laboratory Practice (GLP) standards for quality assurance (Mitsubishi Chemical Safety Institute 2008a) these data could be regarded as the most reliable for environmental risk assessment. The data generated by Rhône-Poulenc Chimie (1977) add value in so far as there seems not to be a significant greater sensitivity of cyanobacteria and diatoms compared to green algae.

#### 4.1.3 Tests with Crustacea

With the freshwater crustacean *Daphnia magna*, data from several acute and chronic test exposures are available. Rufli and Mueller investigated the acute toxicity of 4,4'-MDA against *Daphnia magna* (Rufli and Mueller 1985b) according to OECD Guideline 202 (1981). The exposure time was 24 h, whereas the current guideline requires a 48 h exposure. Due to a resulting odd dose–response behavior (Fig. 4), an  $EC_{50}$  could not be established. The authors reported 24 h  $EC_0$  and  $EC_{100}$  values of < 3.2 and > 100 mg/L, respectively. However, taking the three lowest concentrations only, a more meaningful dose–response relationship becomes evident, and the 10 mg/L exposure would appear to cause about 75 % immobility. Exposure concentrations of this study are based on measured concentrations, but the analytical method was not mentioned in that report. By probit analysis, an  $EC_{50}$  of 5.7 mg/L can be calculated; however, using this reduced data set a 95 % C.I. cannot be derived.

Fujiwara (1982) reported a 24 h  $EC_{50}$  of 2.3 mg/L for *Moina macrocopa*, a common aquatic invertebrate in the Asia-pacific region which tolerates high salinity (95 %-C.I.: 1.8–3.0 mg/L). The test substance was claimed to be extra-pure with no



**Fig. 4** Immobility responses associated with acute (24 h) exposures of 4,4'-MDA to *Daphnia magna* (Rufli and Mueller 1985b)

further details provided. The composition and pH of the test water were described. For each exposure concentration, 4 replicates of 5 animals each were prepared. The reference toxicant potassium dichromate ( $K_2Cr_2O_7$ ) served as positive control. There is no information concerning an analytical method. Therefore, results reported are most likely based on nominal concentrations.

In an OECD Guideline 202 study conducted in compliance with GLP, the acute  $EC_{50}$  for *Daphnia magna* was 2.47 mg/L after an exposure time of 48 h (Mitsubishi Chemical Safety Institute 2008b). After 24 h, the test solutions were renewed (semi-static). The 24 h  $EC_{50}$  was 8.08 mg/L (95 % C. I.: 5.23–12.8 mg/L). After an exposure time of 48 h, the highest concentration at which no effects were observed was 0.2 mg/L, and the  $EC_{100}$  was 200 mg/L. The 95 % C.I. for the 48 h  $EC_{50}$  was 1.27–4.4 mg/L. Concentrations of the test substance were verified analytically with HPLC-UV. The purity of the test-substance was 99.6 %. Another more recent OECD Guideline 202 (OECD 2004) study on the acute toxicity of 4,4'-MDA to *Daphnia magna* was issued in 2011 (Salinas 2011). The 48 h  $EC_{50}$  was determined as 0.35 mg/L (95 % C. I. 0.18–0.71 mg/L), whereas the 48 h  $EC_0$  and 48 h  $EC_{100}$  were 0.22 and 2.2 mg/L, respectively. The purity of the test substance was 98 %. According to HPLC-MS analyses, impurities consisted of an isomer to 4,4'-MDA, as well as some 3-ring MDA and N-methylated by-products. The concentrations of the test solutions maintained over the exposures were verified with HPLC-UV. This test was performed under static conditions.

The acute toxicity of pMDA to *Daphnia magna* was checked in an OECD Guideline 202 test (Salinas 2012). The test substance was characterized by IR, UV, HPLC-MS, GC and  $^1H$ -NMR. The tested product consisted of 55.9 % 4,4'-MDA, 7.1 % 2,4'-MDA, 0.3 % 2,2'-MDA, 26.1 % 3-ring MDA and 9.9 % 4-ring MDA and 0.7 % impurities, from which N-methyl-methylene-4,4'-dianiline and N-formyl-methylene-4,4'-dianiline could be identified. To avoid potential for physical toxicity associated with undissolved particulate matter in test solutions, pMDA was dissolved in peroxide-free THF ( $H_2O_2 \leq 1$  ppm) and the THF was evaporated in a rotating round bottom flask. M4 medium was added to the dried flask and slowly shaken (50 rpm) for 24 h in the dark. Finally, the resulting test solutions were filtered through a 0.2  $\mu m$  membrane. Analyses were performed to determine total dissolved organic carbon (DOC) and 4,4'-MDA concentrations by HPLC-UV. The derived 48 h  $EC_{50}$  of 0.46 mg/L (nominal) or 0.26 mg/L (as measured 4,4'-MDA), indicate that the acute toxicity of pMDA to *D. magna* is in the same order of magnitude as that for pure 4,4'-MDA.

In addition to the aforementioned acute tests with aquatic crustacea, two chronic exposure tests have been conducted to determine potential effects on survival and reproduction. Fujiwara (1982) performed a 14 days chronic test with *Moina macrocopa*. The test period covered three generations of the organism. Animals were not older than 24 h at the beginning of the test. Per test concentration, four replicates with ten individuals each were prepared. The test solutions were renewed every 48 h, and the organisms were fed with unicellular green algae daily. The 14 days NOEC for reproduction was 0.15 mg/L. There is no information concerning

an analytical method; therefore, results reported are most likely based on nominal exposure concentrations.

In 2002, Mitsubishi Laboratories performed a chronic study (21 day) with *Daphnia magna* according to GLP and OECD Guideline 211 (1998); the data were made available in English in 2008 by the International Isocyanate Institute (Mitsubishi Chemical Safety Institute 2008c). Test solutions were renewed daily. The purity of the test substance was 99.6 %, and exposure concentrations were verified using HPLC-UV analyses. In the scope of that test it was observed that the 48 h EC<sub>50</sub> was between 0.019 and 0.06 mg/L; the authors reported a 21 day NOEC of 0.00525 mg/L and a lowest observed effect concentration (LOEC) of 0.0182 mg/L based on the reproduction endpoint.

Concerning acute toxicity and LC/EC50 values for crustacea, *D. magna* is the most sensitive of all species tested with the MDAS, owing to the 48 h EC<sub>50</sub> of 0.019–0.06 mg/L (Mitsubishi Chemical Safety Institute 2008c) and the results of the Salinas (2011) study, yielding a 48 h-EC<sub>50</sub> of 0.35 mg/L. This latter value is not significantly different to that of pMDA (0.46 mg/L), tested in the same laboratory. The highest 48 EC<sub>50</sub> value reported for daphnia is 2.47 mg/L (Mitsubishi Chemical Safety Institute 2008b). Therefore, the MDA acute toxicity to crustacea spreads over two orders of magnitude if results 48h data from acute and chronic tests are considered; focused on data from acute tests only, the difference between 48 h EC<sub>50</sub> values is about 10. Shortcomings in the tests performed were not identified, and impurities are not a likely explanation as this bulk industrial chemical commodity is synthesized via a well known, standard route. The 24 h EC<sub>50</sub> values for K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> were in the range required by OECD Guideline 202. In the Mitsubishi Chemical Safety Institute acute and chronic tests (2008b, c), the water was renewed daily. The only remaining difference identified is the fact that in the chronic test, the daphnids were fed daily with *Chlorella vulgaris*. It remains to be found out whether this difference to the acute test is at least partially responsible for the differences in results.

#### 4.1.4 Test with Vertebrates (Fish)

Rufli and Mueller reported the acute toxicity of MDA against zebrafish (*Danio rerio*) (1985c). The static test was performed according to OECD Guideline 203 (1984). This study resulted in a 96 h LC<sub>50</sub> of 42 mg/L (95 % C.I.: 35–51 mg/L). Results were based on measured concentrations; however neither the analytical method for concentrations verification nor the accurate purity of the test substance was given. Rufli and Mueller (1985d) also reported the acute toxicity of 4,4'-MDA against rainbow trout (*Oncorhynchus mykiss*; formerly *Salmo gairdneri*), following OECD Guideline 203 (1984) under a static exposure procedure. The 96 h LC<sub>50</sub> was 39 mg/L (95 % C.I.: 20–134 mg/L). Results are based on measured concentrations, but again neither the analytical method for concentrations verification nor the accurate purity of the test substance was given.

A further report on the toxicity of MDA against zebra fish is reported by Caspers et al. (1986), following OECD Guideline 203 (1984). The authors report a 96 h LC<sub>50</sub> of 65 mg/L, and 96 h LC<sub>0</sub> and LC<sub>100</sub> values of 40 and 70 mg/L, respectively. The test substance consisted of 99.7 % 4,4'-MDA, 0.25 % 2,4'-MDA and 0.05 % 2,2'-MDA. Maintenance of the exposure concentrations over 96 h was verified using dissolved organic carbon (DOC) analyses.

Munk and Kirsch (1988) reported the acute toxicity of MDA against golden orfe (*Leuciscus idus*). The test procedure followed the German standard DIN 38412 (DIN 1980). Chloro acetamide was used as a positive control. The dissolved oxygen content of the water was throughout > 60 % of the air saturation value, pH was maintained at 8, and the water temperature was held at 20 ± 1 °C. Water hardness and salinity were kept in the required range. Purity of the test substance was claimed to be at least 96 %. The 96 h LC<sub>50</sub> was determined by graphical interpolation to be 53 mg/L. The 96 h LC<sub>0</sub> was 21.5 mg/L and the 96 h LC<sub>100</sub> was 100 mg/L, where derivation of these endpoints is based on nominal concentrations. At higher concentrations, the test substance was noted to have precipitated from solution.

In an English-translated summary report of original studies of the Chemicals Inspection and Testing Institute-Japan, a 48 h LC<sub>50</sub> of 32 mg/L was reported for the orange-red killifish, *Oryzias latipes* (CITI 1992). The testing regime was semi-static, with renewal of the exposure solutions every 8–16 h, and followed the Japanese standard JIS K 0102-1986-71. The Mitsubishi Chemical Safety Institute similarly investigated the acute toxicity of MDA with *Oryzias latipes* in 2002 according to OECD Guideline 203 (1992) and in compliance with GLP. The translated report was issued by the International Isocyanate Institute (Mitsubishi Chemical Safety Institute 2008d). Applying a semi-static method with daily renewal of the test solutions, a 96 h LC<sub>50</sub> of 20.6 mg/L was reported (95 % C.I.: 16.7–25.3 mg/L). These results were based on measured concentrations (HPLC-UV) and the test substance had a purity of 99.6 %.

#### 4.1.5 Tests with Mollusca

The acute toxicity of a pMDA sample containing 60 % 4,4'-MDA was examined using 20 h static aquatic exposures to the Great Pond Snail (*Limnea stagnalis*). The tests were performed in reconstituted river water in the dark at 20 °C, with pH of 8.0 and dissolved oxygen maintained at ≥ 80 % air saturation. A cohort of 6–8 days post-hatch snails was investigated for egg laying performance, and a 1–4 days post-hatch cohort was checked for survival. The LC<sub>50</sub> for the post exposure 4 days survival of young snails was 210 mg/L, and for egg laying performance (fecundity) the LC<sub>50</sub> was 220 mg/L (Rhône-Poulenc Chimie 1977). Aside from this study, no other studies of MDAS with freshwater or marine mollusca are known. Based on this single study, this animal phylum would appear to be less sensitive to the MDAS substances than are the crustacea.

## 4.2 Toxicity to Benthic Organisms

While 4,4'-MDA was under evaluation in the European Union, effects of chemicals to benthic sediment organisms gained interest. This was particularly true for substances highly hydrophobic and/or reactive substances such as the MDAS which have high affinity for sediment and associated organic matter. In a research program of the German Umweltbundesamt (UBA; Environmental Protection Agency), the sediment blackworm *Lumbriculus variegatus* was revealed to be particularly sensitive against primary aromatic amines (Riedhammer and Schwarz-Schulz 2001). As a consequence, 4,4'-MDA was tested against the sediment organisms *Lumbriculus variegatus*, *Chironomus riparius* and *Hyalella azteca*, representing three different taxa of sediment organisms (oligochaete, insects and amphipods) and playing an important role in the aquatic food chain and in organic matter turnover. Data concerning benthic organism are summarized in Table 4.

### 4.2.1 Tests with an Oligochaete

The benthic oligochaete, *L. variegatus* (blackworm) inhabits a wide range of sediment types and is typically present in shallow water sediments of slowly flowing rivers which are rich in organic matter. It is feeding on mainly subsurface organic matter of dead and decaying organisms, and because primary aromatic amines are known to be covalently bound with such organic matter, the potential effects on this organism are of relevance. Egeler and Ginzburg (2001) investigated the toxicity of 4,4'-MDA to *L. variegatus*. The study was conducted in compliance with GLP, but since the now standardized OECD Guideline 225 (2007) for sediment-water *Lumbriculus* toxicity tests was still under development, and

**Table 4** Summary of toxicity of 4,4'-methylenedianiline (MDA) to sediment organisms

Species	Duration	Endpoint and value (mg/L)	References
Oligochaete <i>Lumbriculus variegatus</i>	28 days	NOEC = 25.2 LOEC = 50.3	Egeler and Ginzburg (2001)
Oligochaete <i>Lumbriculus variegatus</i>	28 days	NOEC < 3.75 <sup>a</sup> , NOEC = 30 <sup>b</sup> LOEC = 60 <sup>b</sup>	Egeler (2002)
Insect larvae <i>Chironomus riparius</i>	28 days	NOEC = 500 LOEC = 1000	Egeler and Gilberg (2005a)
Amphipod <i>H. azteca</i>	28 days	NOEC = 41.4 <sup>c</sup> NOEC = 90.9 <sup>d</sup> LOEC = 90.9 <sup>c</sup> LOEC = 200 <sup>d</sup>	Egeler and Gilberg (2005b)

<sup>a</sup>Based on statistics (see text)

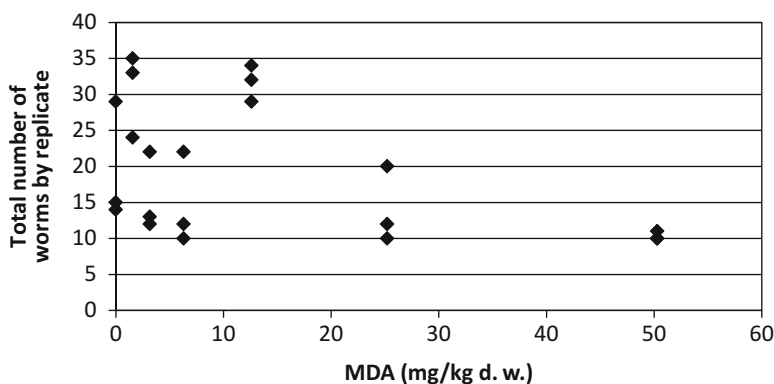
<sup>b</sup>Biological interpretation (see text)

<sup>c</sup>Survival

<sup>d</sup>Biomass

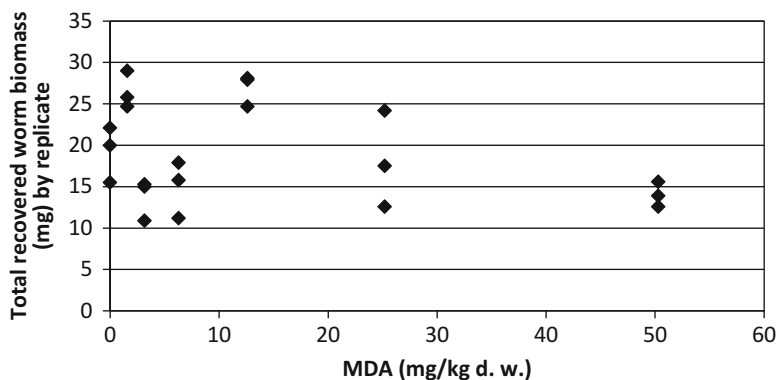
because *Lumbriculus* turned out to be most sensitive sediment organism against MDA with a non-monotonic dose–response, a more detailed explanation on test design and results will be provided in the following section.

In a first test with *L. variegatus*, the artificial sediment consisted of 75 % quartz sand, 20 % clay and 5 % sphagnum peat. The peat content was chosen to represent a moderate content of organic matter as indicated at that time. Further, between 0.05 and 1 % pure  $\text{CaCO}_3$ , 0.4 % TetraMin<sup>®</sup> fish food (Tetra Werke, Melle, Germany) and 46 % water were added per sediment dry weight. While the test was running, further TetraMin<sup>®</sup> was added on the surface of the sediment at the beginning of weeks 2, 3 and 4. Reconstituted water was used according to OECD guideline 203 (1992) Annex 2. During the test, the temperature was maintained at  $20 \pm 2^\circ\text{C}$ , and a 16/8 h light (100–1000 Lux)/dark cycle was applied. Air was slowly passed through the aqueous layer at one bubble per second. As MDA can bind covalently to sediment, spiked sediment was allowed to equilibrate for 2 days before the test organisms were added. This equilibration time was chosen to ensure that added organisms were exposed against both organic matter-bound and freely dissolve MDA. Per dose group, three replicate exposures were prepared with ten synchronized worms added to each of the 1 L test vessels, filled with equilibrated sediment and reconstituted water. Nominal concentrations of 4,4'-MDA (purity 97.9 %) were 0, 1.6, 3.1, 6.3, 12.6, 25.2, 50.3 and 100.7 mg/kg sediment dry weight. The spiking solutions, overlying water, and sediment pore-water were analyzed by HPLC-UV. During the test, the dissolved oxygen in the overlying water was at least 74 % of the air saturation value, and the pH was kept between 6.0 and 7.0. At the end of the 28 days exposure period, the number of worms and total worm dry weight were analyzed by ANOVA and Dunnett's *t*-test. In the control group, mortality did not exceed 7 %. For reproduction (total number of worms and number of regenerated worms), biomass, and number of worms with new posterior and anterior end, a clear dose–response pattern could not be observed (Figs. 5–7). Due to the reactivity of MDA with organic matter, and possibly also due to its biodegradation in the sediment-water test system, its concentration in the aqueous phase declined over

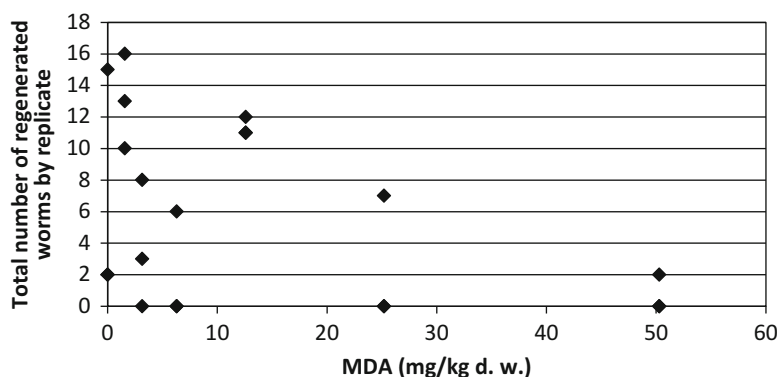


**Fig. 5** Representative plot of number of *L. variegatus* worms recovered from replicate 4,4'-methylendianiline (MDA) sediment-water exposure vessels after 28 days (adapted from Egeler and Ginzburg 2001)





**Fig. 6** Representative plot of total worm biomass recovered from replicate 4,4'-methylenedianiline (MDA) sediment-water exposure vessels after 28 days (adapted from Egeler and Ginzburg 2001)



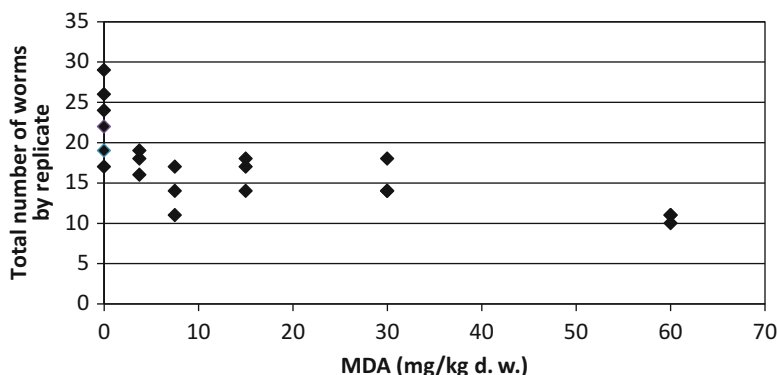
**Fig. 7** Representative plot of total of total number of *L. variegatus* worms with new posterior/anterior ends recovered from replicate 4,4'-methylenedianiline (MDA) sediment-water exposure vessels after 28 days (adapted from Egeler and Ginzburg 2001)

time. The mean recovery of MDA was about 4 % of that initially applied after 28 days. Based on nominal concentrations, the NOEC and LOEC values for biomass were 50.3 and 100.7 mg/kg d. w., respectively, whereas for reproduction the values were 25.2 and 50.3 mg/kg d. w. Based on measured concentrations after 28 days, the NOEC and LOEC were 2.1 and 4.1 mg/kg d. w. against biomass and 1.0 and 2.1 mg/kg d. w. for reproduction. For lower nominal concentrations, extractable MDA was not detectable at the end of the test period (<0.1 mg/kg d. w.). In freshly-spiked sediment, recovery of MDA was 82–102 % of the applied concentration. Therefore, the extraction and analytical methods as such were effective. The disappearance of the MDA in these test systems was most likely attributable to its irreversible binding to organic matter of the formulated sediment, and perhaps also biodegradation in both sediment and water layers. In the previously described test with *L. variegatus*, it was speculated the iterative feeding with

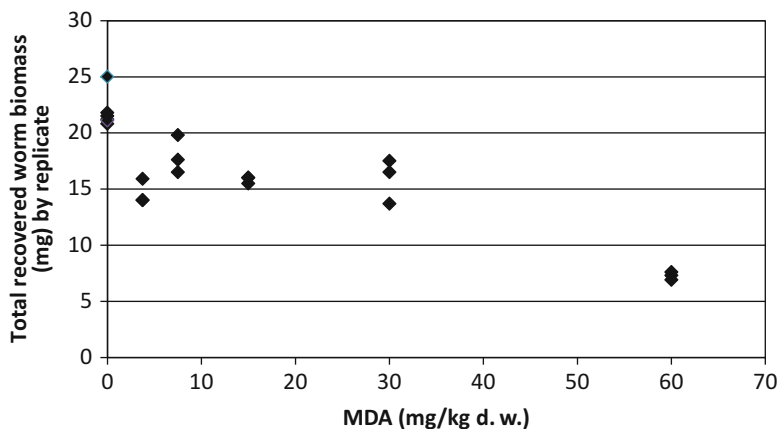
TetraMin<sup>®</sup> could predispose the test organisms towards selective feeding on this uncontaminated food versus the MDA-associated sediment organic matter. In such a case, the true toxicity of MDA-associated sediment organic matter may be misrepresented. A second study was performed to check whether sediment amended with an organic matter food source— here nettle powder (*Urtica* sp.)— instead of semi-continuous feeding would deliver different results (Egeler 2002). Nominal concentrations of 4,4'-MDA (purity 97.9 %) were 0, 3.75, 7.5, 15, 30 and 60 mg/kg sediment dry weight. Due to high variability among replicates observed in the previous test, six replicates were used for the control and three replicates for each exposure concentration. The test solutions used to spike the formulated sediment with MDA were verified by HPLC-UV, but further analyses of pore-water and overlying water was deemed not necessary due to the reactivity of MDA to sediment organic matter.

A dose–response curve could not be fitted to the data due to high variations between dose groups (Figs. 8 and 9). For the total recovered biomass endpoint, the lowest test concentration of 3.75 mg/kg d. w. was the LOEC after 28 days. For reproduction, the 28 days NOEC was 3.75 mg/kg and the 28 days LOEC was 7.5 mg/kg d. w. sediment. For regenerated worms with new anterior and posterior ends, the effect of MDA was more prominent. In the control vessels, the number of complete new worms ranged from 2 to 8. In all exposure vessels, there was at maximum one complete new worm.

The results from these two tests with *L. variegatus* would indicate that the feeding regimen employed can influence the derived effective concentrations for biomass, reproduction, and regeneration of this sediment-feeding worm. The test which employed feeding via the overlying water appeared to result in higher effective concentrations for the MDA substance. However, it cannot be determined how difference in feeding modes among these two studies could influence other factors such as microbial activity (biodegradability MDA) and ammonia production



**Fig. 8** Representative plot of total of total number of *L. variegatus* worms recovered from replicate 4,4'-methylendianiline (MDA) sediment-water exposure vessels after 28 days (adapted from Egeler 2002)



**Fig. 9** Representative plot of total worm biomass recovered from replicate 4,4'-methylenedianiline (MDA) sediment-water exposure vessels after 28 days (adapted from Egeler 2002)

as confounding toxicant in these sediment-water test systems. Nevertheless, this benthic oligochaete was shown to be most sensitive to MDA in both tests, compared to other species evaluated.

#### 4.2.2 Tests with an Insect Larvae

Larvae of the harlequin fly (*Chironomus riparius*) are living in and on sediments, and are feeding on organic matter deposited in the sediment during this life cycle stage. As such, they too are a relevant test organism for assessing potential hazard of substances such as the MDAS which bind with sediment organic matter. In an OECD Guideline 218 study, Egeler and Gilberg (2005a) tested the chronic toxicity of 4,4'-MDA (purity 97.9 %) to *C. riparius*. Prior to test substance addition, the sediment was spiked with 0.4–0.5 % stinging nettle powder as the sole food source. As West et al. (2004) discovered, the spiking of sediment with nettle powder can produce significant amounts of ammonia. As a result, in this study a tighter control of pH values was maintained to maximize speciation of any ammonia produced as the less toxic ammonium ion. In addition, aeration of the overlying water layer was doubled from that employed in the *L. variegatus* tests from one to two bubbles of air per second, so that any un-ionized ammonia (gas) would be more readily expelled. Larvae were added 2 days after spiking the sediment with MDA. This procedure ensured that part of the test substance is already bound to sediment, while another part is still available in the aqueous phase. Nominal test concentrations of 4,4'-MDA (purity 97.9 %) were 62.5, 125, 250, 500 and 1000 mg/kg d. w. sediment. Due to the experience with previous tests, MDA was analyzed in stock solutions only, but not in sediment and overlying water. The pH was kept in the range of 7.3–8.3 by occasional addition of diluted HCl (pH up to 9 would be acceptable

according to the guideline). The ammonium content in the overlying water ranged from 0.2 to 16.1 mg/L in the exposures employed in this test. For the emergence ratio endpoint, the 28 days NOEC was 500 mg/kg dry weight, and 1000 mg/kg d. w. was the 28 days LOEC. For the development rate endpoint, 1000 mg/kg d. w. was determined to be the 28 days NOEC. Thus, the larvae of the *C. riparius* insect appear to be much less sensitive to MDA than is the oligochaete *L. variegatus*.

### 4.2.3 Test with an Amphipod

A third organism has been tested with chronic exposures to MDA, to enable an analysis of species sensitivity across three taxa of benthic organisms and their associated feeding modes. The amphipod crustacean *Hyalella azteca* shows some subsurface deposit feeding, but has a stronger affinity to feeding on organic matter deposits at the surface of sediments than the previously describe benthic organisms. The *H. azteca* organism is very sensitive to ammonia (Whiteman et al. 1996); the sensitivity depends on the pH value of the test medium. At a nominal concentration of 50 mg/L  $\text{NH}_4^+$  (148 mg/L  $\text{NH}_4\text{Cl}$ ) in the overlying water, all exposed amphipods survived a 4 days exposure period in M4 medium at pH values not higher than 8.5. When the pH value was raised to 9, all exposed organisms died within 24 h (Egeler 2004).

Egeler and Gilberg (2005b) tested the chronic toxicity of 4,4'-MDA to *H. azteca*. The testing regime was performed in compliance with GLP and followed OECD Guideline 218 (OECD 2004). The chironomus test conditions were adapted to testing with *H. azteca*, as an OECD guideline for testing with this organism does not exist. In this adapted test, the amount of stinging nettle powder incorporated into the sediment was reduced from the guideline recommended 0.5 % d.w. to 0.25 % d.w. This was thought to reduce potential for ammonia formation from this N-containing organic matter, and 0.25 % cellulose (which contains no nitrogen) was added to replace amount of eliminated nettle. These sediment-incorporated food sources were equilibrated in the test system before addition of the test substance. Nominal concentrations of 4,4'-MDA (purity 97.9 %) were 8.5, 18.8, 41.3, 90.9 and 200.0 mg/kg sediment d. w.; spiking solutions were analyzed by HPLC-UV, but no analysis was performed for the sediment and the overlying water due to the known reactivity of MDA to sediment organic matter. After a 2 day equilibration period organisms were added, and pH was controlled daily between 7.4 and 8.3 by occasional addition of dilute HCl. After 28 days, the NOEC and LOEC for survival were 41.3 and 90.9 mg/kg d. w., respectively. With regard to the amphipod length and total biomass, the 28 days NOEC and 28 days LOEC were 90.9 and 200 mg/kg d. w., respectively.

#### 4.2.4 Additional Tests on Feeding Mode and Ammonia Formation

Over the course of the aforementioned testing of MDA with benthic organisms, the potential confounding of results from feeding of the organisms, the feed types used, and potential for associated ammonia formation from decay of the food was questioned. Additional studies were conducted to improve the understanding of how these test parameters may have influenced results and interpretations of the aforementioned tests. West et al. (2004) reported that spiking of sediment with nettle powder (*Urtica* sp.) at the beginning of formulated sediment tests results in a stronger and earlier increase in ammonia concentration than feeding via the overlying water with semi-continuous addition of TetraMin®. The discovery of these feeding-related influences on ammonia production prompted further investigation using a factorial design study to investigate the influence of different feeding parameters on the water quality in sediment tests with pre-spiked sediments (Egeler and Gilberg 2005c). The food spiked into the sediment was either *Urtica*: cellulose = 1:1 (wt:wt) or cereal leaves (wheat; *Triticum aestivum*), test-substance (4,4'-MDA, 97.9 %) was added or not (10 mg/kg d. w.), aqueous medium was either M4 (Elendt medium) or a pH-reduced medium (by reduction of NaHCO<sub>3</sub>), and organisms added were either the blackworm *L. variegatus* or the amphipod *H. azteca*. The tests were run for 28 days. For *L. variegatus*, cereal leaves was shown to be the best food source concerning total number of worms and biomass. The *urtica* powder/cellulose combination food source resulted in a significant reduction of biomass, especially when the M4 medium was modified. For *H. azteca*, survival and length were not influenced by any of the feeding factors evaluated, but the biomass was significantly reduced when the modified M4 medium was used. The ammonia concentration in the pore water and overlying water was significantly higher when cereal leaves were used as food source. The presence of MDA had no measureable influence on the ammonia content. This can be regarded as an indication that nitrification is not inhibited by up to 10 mg/kg d. w. MDA in the formulated sediment.

In the previously-described second test with the blackworm *L. variegatus* (Egeler 2002), pH values were taken at day 1 and 28 and found both to be below pH 8. However, pH values were not recorded in between and ammonia was not monitored. In sediment spiked with nettle powder, the pH exceeded a value of 8 after 48 h, and then declined to levels below 8 after 11 day, influenced by addition of diluted HCl. Ammonia, however, achieved the highest concentration at day 4 of the experiment (West et al. 2004). The authors further demonstrated that ammonia formation is less a problem in case of semi-continuous feeding with TetraMin®. As a result, the lower NOEC and LOEC in the second study with *L. variegatus* are probably attributable to interference by ammonia. Although the number of worms with new posterior and anterior end seems to be most sensitive parameter in the second study, results from the first study demonstrated that this endpoint suffers from a high variability even in the control. As a result, this endpoint is not suitable

for the interpretation of the toxicity of MDA to *L. variegatus* under the test design employed.

Sediment tests performed with MDA generally showed a high variability in outcomes. This was at least partly attributable to the confounder ammonia production. In addition, MDA can be regarded as a “difficult substance” for sediment testing. For example, in an OECD Guideline 308 study simulating the fate of radiolabeled MDA in a surface water and sediment system (Schaefer and Ponizovsky 2013), there was no MDA detectable in the aquatic layer 7 days after test initiation. After 100 day, 5.7 % of the MDA was transformed to CO<sub>2</sub>, about 6 % MDA metabolites were detectable in the water, 12 % MDA products were extractable from the sediment and 90 % remained unextractable (retrievability about 114 %). Especially in the first few days after addition to the sediment, the MDA suffers rapid transformations against the time frame of the chronic sediment tests. The parent substance and the metabolites are assumed to show different toxicity against the sediment organisms. The design as used was chosen as such because it is not known whether MDA itself or one of the transformation products is most critical. The variability in test outcomes observed seems to be attributed to subtle variations in conditions of these dynamic test systems.

### 4.3 Toxicity to Terrestrial Organisms

The reactivity of the MDAS with natural organic matter indicates the potential for their association with surface soils should direct exposures occur as a result of spillages, or indirect exposures such as a hypothesized hydrolysis and deposition of atmospheric emissions of MDI substances (Government of Canada 2014). Data with soil organisms are summarized in Table 5.

#### 4.3.1 Tests with Soil Bacteria

Soil bacterial serve a critical role in the cycling of nitrogen in the environment. The decay of nitrogen-containing organic matter can result in the release of ammonia, and this ammonia can then be oxidized by specific nitrifying bacteria to nitrite and nitrate. Because several aromatic amine substances were shown to behave as inhibitors of these nitrifying bacteria in soil (Zhang et al. 2010), the potential for MDAS to have similar effect was evaluated using a modification to the OECD Guideline 216 (2000). The standard version of this OECD test recommends that plant material is added to soil and to check for the formation of nitrate. The principle of this test is that as heterotrophic soil bacteria decompose this plant material to release ammonia, the amount of nitrate formed from this ammonia is dependent upon the activity of the specific denitrifying bacterial population in the soil. A reduction in the rate or total mass of nitrate production in soil treated with the test substance, compared to that in an untreated control soil, is interpreted as

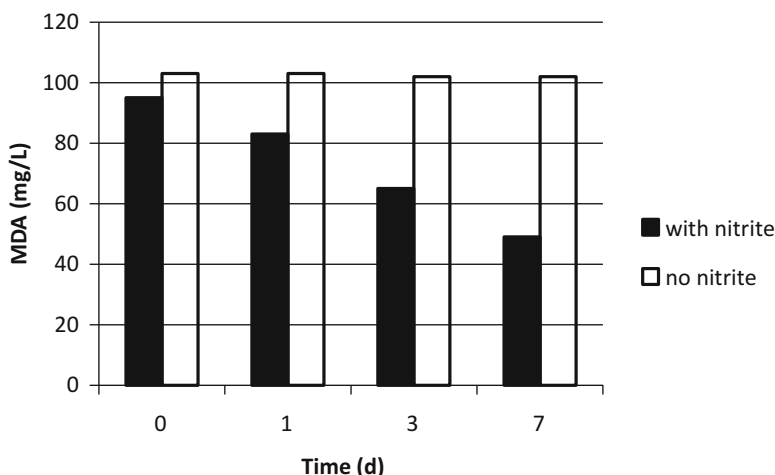
**Table 5** Summary of acute and chronic toxicity studies for 4,4'-methylenedianiline (MDA) with soil organisms

Organism	Endpoint	Value (mg/kg d. w.)	References
Oats <i>Avena sativa</i>	17-days growth	EC <sub>50</sub> = 353	Van der Hoeven et al. (1992a)
Lattuce <i>Lactuca sativa</i>	17-days growth	EC <sub>50</sub> = 128	
Radish <i>Raphanus sativus</i>	6-days emergence	EC <sub>20</sub> = 96 <sup>a</sup> EC <sub>50</sub> = 331 <sup>a</sup>	Kim et al. (2002)
Earthworm <i>Eisenia fetida</i>	14 days mortality	EC <sub>50</sub> = 444	Van der Hoeven et al. (1992b)
Earthworm <i>Eisenia fetida</i>	56 days reproduction/ mortality	EC <sub>50</sub> = 333	Moser and Hamberge (2012)
		EC <sub>10</sub> = 11	
		LOEC = 18	
Springtail <i>Folsomia candida</i>	Reproduction 28 days	NOEC = 562	Moser and Schott (2011)
		LOEC = 1000	
Bacteria Nitrification in soil	NH <sub>4</sub> <sup>+</sup> depletion; NO <sub>3</sub> <sup>-</sup> formation	NOEC = 1000	Schwarz (2013)
Nitrification in activated sludge	NH <sub>4</sub> <sup>+</sup> depletion	IC <sub>50</sub> = 61 mg/L	

<sup>a</sup>In water

potential inhibition of these nitrifying soil bacteria. However, recognizing that the MDAS are reactive aromatic compounds, and nitrite, formed transiently during nitrification of ammonia, may react directly with the MDAS by forming azo-compounds or nitroso-aromatics. If such reactivity were to occur under conditions of the standard OECD 216 test, the resulting reduction in nitrate formation may be mis-interpreted as inhibition of nitrifying bacteria. In a screening study to examine potential for this reactivity, (unpublished data of BASF Polyurethanes, Gericke 2010) the stability of 4,4'-MDA was observed in an aquatic solutions at pH = 6 in the presence and absence of equimolar amounts of sodium nitrite; the decay of nitrite was not investigated. The solutions were incubated at about 22 °C in the dark. The results of this screening test, shown in Fig. 10, indicated an instability (reactivity) of 4,4'-MDA in the presence of nitrite under these slightly acidic conditions. In the presence of nitrite, the initially colorless 4,4'-MDA solution became yellow after 7 days.

With this knowledge, an OECD Guideline 216 study was performed in a modified way (Schwarz 2013), where instead of plant material the soil was spiked with about 75 mg ammonium sulfate per 5 g dry soil. Over the test period, both the decline of ammonium and the formation of nitrate were monitored. The soil was spiked with 0, 10, 20, 50, 125, 250, 500 or 1000 mg 4,4'-MDA per kg soil (d. w.). The EC<sub>10</sub> value was shown to be above 1000 mg/kg after 14 and 28 days in terms of ammonia depletion, as well as in terms of nitrate formation. Only after 7 days, the EC<sub>10</sub> was between 500 and 1000 mg/kg, indicating a potential minor and transient

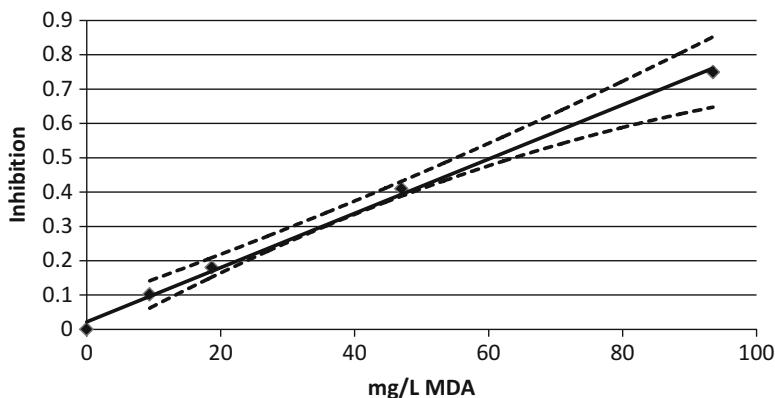


**Fig. 10** Time-dependent concentrations of 4,4'-MDA in aqueous solution in the presence (*black bars*) and absence (*white bars*) of nitrite at pH = 6

effect on the soil bacteria. The amount of MDA measured in aqueous soil extracts taken for nitrate analyses was shown to rapidly decline and was about 14–60 % of the initial lowest and highest MDA concentrations tested, respectively, at the beginning of this test. At the end of the test (28 days), recoveries of the MDA were < 10 % of their initial nominal values. This result is in line with observations in the previously discussed sediment tests with MDA, where an irreversible absorption of MDA to soil organic matter along with biodegradation are the most likely explanation disappearance from the test systems. So, a limited inhibition of nitrification was observable only at the beginning at the highest soil load (1000 mg/kg d. w.), when some freely available MDA was likely still available. The quantitative formation of nitrate further indicates that transiently-formed nitrite was not significantly reacted with MDA, as was shown to be the case in pH 6 aqueous solution (Gericke 2010).

The ISO 9509 test (2006) can be used to check the nitrification inhibition in activated sludge. Initial cursory tests were inconclusive as 17 mg/L seemed to be the IC<sub>50</sub> concerning ammonium decay, but it was difficult to discern even a 10 % inhibition of nitrate formation (Schupp 2014). Therefore, a definitive test was performed with activated sludge of the municipal STP of the city Steinfurt, Germany. The sludge (3 g solids per liter, dry wt.) was spiked with ammonium sulfate and 0, 9.4, 18.7, 47 and 93.5 mg/L 4,4'-MDA (98 % pure). The N-Allyl-thiourea substance, a known inhibitor of nitrification, served as control. The nutrient solution contained 2.71 g/L NH<sub>2</sub>SO<sub>4</sub> and 5.47 g/L NaHCO<sub>3</sub> and was diluted 1:10 for the test runs. Tests were run in duplicate. After 4 h incubation at 20 °C, samples were taken, filtered through a 0.46 µm membrane, and ammonium ions were analyzed photometrically after reaction with K<sub>2</sub>HgI<sub>4</sub>. A blank experiment proved that MDA does not interfere with this photometrical method. In this test, MDA shows moderate





**Fig. 11** Inhibition of ammonium nitrification by MDA in an ISO 9509 test

nitrification inhibition with  $IC_{50} = 61$  mg/L (Fig. 11). HPLC-UV analysis of the 47 mg/L solution after the test showed that MDA was neither absorbed on sludge nor did it suffer other routes of decay.

#### 4.3.2 Toxicity to Terrestrial Plants

The potential effect of MDA on the emergence and growth of monocot (oat, *Avena sativa*) and dicot (lettuce, *Lactuca sativa*) vascular plants was investigated according to OECD Guideline 208 (Van der Hoeven et al. 1992a). The purity of the test substance was 99.5 %. Oat was sown at 1 cm depth, and lettuce on the surface of a semi-natural soil. The soil was previously spiked with sand that was coated with MDA. For the latter, MDA was dissolved in acetone, the sand soaked with this MDA-solution, and the acetone was then evaporated under a stream of nitrogen. Tests were run for 17 days and five replicates at ten seeds per concentration (0, 3.2, 10, 32, 100, 320 and 1000 mg/kg d. w. soil, nominal concentrations). Short-term endpoints were associated with concentrations at which no effects were observed, and the lowest concentration at which effects were observed by pair-wise binomial test ( $2 \times 2$  contingency table).

For the emergence endpoint, concentrations were defined as 320 and 100 mg/kg d. w. at which no effects were observed for 17 days exposures to oat and lettuce, respectively. For growth, the corresponding concentrations showing no effects were 100 and 10 mg/kg d. w. for oat and lettuce, and the corresponding  $EC_{50}$  values were 353 and 128 mg/kg d. w., where these endpoint values are based on nominal concentrations. The dicotyledonous lettuce, therefore, is somewhat more sensitive to 4,4'-MDA than the monocotyledonous oat.

In another seed germination test, Kim et al. (2002) investigated the influence of 4,4'-MDA dissolved in water and poured onto filter paper in petri-dishes on the germination of radish seeds. The precise species, not mentioned in the paper,

probably was *Raphanus sativus*. After 6 days at 20 °C, germination was assessed visually. The reliability of this test may be questioned, as the authors claim to have used MDA solutions up to 1 % MDA (10 g/L) which is clearly beyond the water solubility of MDA (Table 2). Data were presented in graphs only, and germination is estimated to be 86, 48, 29, 14, 5 and 0 % at 0.01, 0.02, 0.06, 0.1, 0.5 and 1.0 % MDA in water, respectively. If the two highest concentrations are omitted due to potential insolubility of MDA, the 6 days EC<sub>50</sub> is interpreted to be 331 mg/L, and a 6 days EC<sub>20</sub> is 96 mg/L is estimated by logit analysis.

### 4.3.3 Toxicity to the Earthworm *Eisenia fetida*

Van der Hoeven et al. (1992b) investigated the acute toxicity of 4,4'-MDA against *E. fetida* after 14 days exposure according to OECD Guideline 207 (1984), using nominal test concentrations of 0, 18, 32, 56, 100, 320 and 560 mg/kg d. w. applied to five replicates per concentration. The 4,4'-MDA (99.5 % purity) was dissolved in acetone and added to artificial soil. Subsequently, the acetone was evaporated under a stream of nitrogen. The 14 days LC<sub>50</sub> was determined to be 444 mg/kg d. w.

Moser and Hamberge (2012) investigated the effect of 4,4'-MDA on the reproduction of *E. fetida* according to OECD Guideline 222 (2004) and in compliance with GLP. Stock solutions of MDA (purity 98 %) were prepared in ethanol, and the concentrations were verified by HPLC-UV. Defined volumes of the MDA solutions were added to quartz sand, and the ethanol was evaporated. The coated quartz sand was used incorporated into the artificial soil, containing 5 % sphagnum peat. Nominal concentrations were 18, 32, 56, 100, 180 and 320 mg/kg d. w., with four replicates used at each concentration and ten worms per replicate. Worms were fed with cow manure which was free of contamination and veterinary pharmaceuticals. At day 56, test vessels were analyzed. For the mortality endpoint, the 56 days NOEC was 180 mg/kg d. w., the 56 days EC<sub>10</sub> was 92.06 mg/kg d. w., and the 56 days EC<sub>50</sub> was 333 mg/kg d. w. soil. For adult biomass endpoint, the 56 days NOEC and 56 days LOEC values were 32 and 56 mg/kg d. w., respectively. For reproduction, the 56 days LOEC was 18 mg/kg d. w. As no NOEC (reproduction) could be derived the 56 days EC<sub>10</sub> of 11.2 mg/kg (95 % C.I.: 0.6–21.3 mg/kg d. w.) was statistically determined instead.

### 4.3.4 Toxicity to the Collembolan Species *Folsomia candida*

The effect after 4 weeks of exposure to 4,4'-MDA on the collembolan species *F. candida* was investigated by Moser and Schott (2011) according to OECD Guideline 232. The 4,4'-MDA test substance (98 % pure), dissolved in ethanol, was spiked to artificial soil containing 5 % sphagnum peat. Spiking solutions were analyzed by HPLC-UV. Nominal concentrations were 56.2, 100, 178, 316, 562 and 1000 mg/kg d. w. soil. For adult mortality, there was no clear dose–response; the highest mortality of 20 % was observed at 316 mg/kg; for reproduction, the 28 days

NOEC was 562 mg/kg and the 28 days LOEC was 1000 mg/kg d. w. The reference substance boric acid generated the expected results.

#### 4.3.5 Acute Toxicity to Birds

Hurlbut et al. (1983) report an acute LD<sub>50</sub> of 148 mg/kg for redwinged blackbirds (*Agelaius phoeniceus*), fed with grains containing 4,4'-MDA. Data on purity of the substance and stability of MDA in/on the food are not provided, and many experimental details are lacking. The reliability of the data generated, therefore, is questionable.

### 4.4 Potential for Endocrine-Modulating Effects and Brief Overview of the Toxicity to Mammalia

A review of data on endocrine activity of MDA was issued by Jaeger and Collins (2011) and Jaeger et al. (2012). A summary of the reviewed findings is presented as follows:

In yeast receptor binding studies, 4,4'-MDA and pMDA showed neither estrogenic nor androgenic activity and no anti-estrogenic activity in the dose range of  $10^{-7}$  to 0.1 mmol/L; however, 4,4'-MDA and pMDA showed apparent anti-androgenic activity at concentrations of  $10^{-3}$  and  $10^{-2}$  mmol/L, respectively (Kolle and Landsiedel 2010a, b, c, d). Since MDA has potential to react directly with the endogenous hormone (testosterone) and with proteins which are the construct of the androgen receptor, the apparent anti-androgenic activity associated with high MDA concentrations could be an artifact of this reactivity and not necessarily indicative of receptor-mediated antagonism.

In a subacute oral study, castrated female rats received 150 and 200 mg/kg MDA for up to 14 days (Tullner 1960). The body weight decreased whereas uterine, thyroid and adrenal weights increased. The adrenals showed extensive lipid accumulation, and thyroid follicles contained little or no colloid.

Intravenous administration of 50 and 100 mg/kg MDA caused a transient reduction in 17-hydroxycorticoid output in the dog (Tullner 1960).

In ovariectomized rabbits, subcutaneous administration of MDA caused a progestational response. However, in ovariectomized and adrenalectomized rabbits, MDA did not cause a response, whereas subcutaneous progesterone did. Cortisol did suppress the progestational response to MDA, but not that due to progesterone (Tullner 1960).

Rats received 0, 80, 400 or 800 ppm MDA in drinking water for 3 months (Ciba-Geigy 1982). Besides hematotoxic and hepatotoxic effects, thyroid follicular epithelial cells were hypertrophic, and the glandular structures showed a diffuse hypertrophy and colloid depletion. In another subchronic drinking water study

with rats, similar effects were reported (DHHS NTP 1983). In both studies, gonads and accessory organs were not affected.

In mice, MDA caused mainly hepatotoxicity; goiters with papillary hyperplasia and vacuolization of the colloid were observed in one high dose male and female animal (DHHS NTP 1983).

In chronic drinking water studies, MDA caused thyroid follicular cell adenomas and carcinomas in rats and mice (DHHS NTP 1983). This effect might be attributable to irreversible inhibition of thyroid peroxidase by the reactive MDA substance at high dose levels. In a later study, MDA was shown to be an effective inhibitor of hog thyroid peroxidase (TPO) in vitro with an IC<sub>50</sub> of 0.06  $\mu$ M (Freiberger 1994). However, direct genotoxicity of MDA in thyreocytes was demonstrated by a positive comet assay (Martelli et al. 2002).

## 5 Summary

Concerning chronic toxicity, *D. magna* is the most sensitive species tested against MDA aquatic exposures, with a 21 day-NOEC of 0.00525 mg/L. Exposure of daphnids takes place via the aquatic phase. Other species of the same phylum (*Arthropoda*) appear to be less sensitive albeit with exposures via soil or sediment, with a 28 days-NOEC of 562 mg/kg d. w. soil (*F. candida*) and 41.3 mg/kg d. w. sediment (*Hyalella azteca*), for reproductive and survival endpoints, respectively. Also for acute toxicity, *D. magna* is more sensitive than the other species, with an 48 h-EC<sub>50</sub> that spreads over two orders of magnitude, ranging from 0.019 to 2.7 mg/L; the reason for this large difference is not clear at the moment. Data laying at both ends of the range are from the same lab, testing the same charge of MDA, and the only difference visible so far is the feeding mode. That is, daily feeding with algae resulted in an 48 h EC<sub>50</sub> of 0.019–0.06 mg/L, whereas the 48 h EC<sub>50</sub> from the acute test with no feeding is 2.47 mg/L. Future tests might investigate whether intake of MDA via the food chain results in a significantly higher body burden in the daphnids than intake via water, only. Fish show a more uniform reaction to MDA, with 96 h-LC<sub>50</sub> values ranging from about 20 to 60 mg/L; chronic data for fish are not available. Acute toxicity data for algae and cyanobacteria are in the range of 1–10 mg/L; based on growth-rate, the 72 h-NOEC or EC<sub>10</sub> of MDA to algae is 0.3–9.3 mg/L.

For sediment organisms, the blackworm *L. variegatus* shows the highest sensitivity to MDA with NOEC values between  $\leq 3.75$  mg/kg and 30 mg/kg d. w., followed by the amphipod *H. azteca*. The higher sensitivity of *L. variegatus* in the second study compared to the first study is obviously attributable to the different feeding regimes (semi-continuous feeding against pre-spiked sediment). One argument might be that semi-continuous feeding allows the organisms to avoid the contaminated food. However, a change from semi-continuous feeding to sediment pre-spiked with nettle powder (*Urtica* sp.) results in an earlier and much stronger increase in ammonia concentration in the system. This became apparent after both

studies on *the blackworm* were finalized. The ammonia 96 h-EC<sub>50</sub> for the *blackworm* is 0.69 mg/L at pH = 8.2, and the 96 h-EC<sub>10</sub> at pH = 8.2 is 0.33 mg/L (Hickey and Vickers 1994). As a result, the lower NOEC and LOEC in the second study with *L. variegatus* are probably attributable to interference by ammonia.

MDA binds irreversibly to soil and sediment which may explain the general, but not uniform lower sensitivity of soil and sediment organisms against aquatic organisms. The intrinsic toxicity of MDAS, beyond that attributed to a baseline narcosis mode of action, is imparted by the reactivity of the primary aromatic amine groups. Thus, reaction of these amine groups with organic matter (macromolecules) would be expected to coincide with reduced reactivity, toxicity, and bio-availability. However, species with intense soil or sediment contact (*L. variegatus* and *E. fetida*) show in general lower NOEC values than those organisms with less direct contact (3.75 and 11 mg/kg d. w., respectively). On the one hand it may be hypothesized that this intense contact to soil-bound MDA is one reason for the higher sensitivity; on the other hand, metabolic capacity against MDA of the organisms tested is unknown at this point in time and might as well explain differences in species sensitivity. For plants there are only acute data available; for the terrestrial species tested, *L. sativa* is more sensitive to MDA than *E. fetida*.

Limited aquatic data available so far do not indicate that the toxicity of pMDA is different from that of MDA. In addition, the limited set of data generated with the marine *M. macrocopa* (crustacean, acute and chronic test), *N. fustulum* (diatom) and *V. fischeri* (bacteria) do not indicate that sea water organisms are more sensitive to MDA than fresh water organisms.

In mammals, MDA is unlikely to interact directly with the endocrine-mediated homeostatic, growth, and developmental pathways; interaction with the adrenergic system cannot be ruled out, and apparent effects of MDA on the thyroid hormone system have been demonstrated. MDA inhibits the thyroid peroxidase enzyme which might contribute to the thyroid gland tumors observed in chronic studies with rats and mice. Some anti-androgenic activity in vitro did not prevail in the in vivo studies with rats and mice. Main target organs in mammals are the liver and the thyroid gland. MDA shows genotoxicity in different test systems and is a carcinogen by GHS category 1b.

## 6 Conclusion

The toxicity of 4,4'-Methylenedianiline, its isomers and homologues (MDA substances; MDAS) against organisms in the environment was iteratively tested over at least four decades. This review underlines the importance of accurate substance identification and analyses that accompany ecotoxicity tests for substances which can react with or in environmental compartments. Especially for the sediment and soil compartment that absorb MDA reversibly and irreversibly, the influence of these processes on the biological availability and toxicity of MDA and its products

requires careful consideration. Sediment tests with MDA were performed at a time where the increase in knowledge concerning reactive substance was on a steep slope, and the guideline for *L. variegatus* was under development. This, together with the dynamics of the MDA molecule in the sediment, may explain variations in findings of the sediment tests. There is a remarkably high variation in toxicity values in acute daphnia studies. Whereas most values are in the range of 0.46–5.7 mg/L which represent the typical variation in ecotoxicology there is one study showing significant lower EC50 values in the range of 0.019–0.06 mg/L, thus indicating that the factors governing ecotoxicity of MDAS to daphnids is currently not fully understood.

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