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Confirmation of combinational effects of calcium with other metals in a paper recycling mill effluent on nematode lifespan with toxicity identification evaluation method

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Abstract

We used toxicity identification evaluation (TIE) method to confirm the combinational effects of identified toxic metals in a paper recycling mill effluent in inducing the decreased lifespan in nematode *Caenorhabditis elegans*. Exposure to Ca + Al caused more severely decreased lifespan than that exposed to Ca, or Al; and exposure to Ca + Fe induced more severely decreased lifespan than that exposed to Ca, or Fe. Exposure to Ca+Al+Fe caused more severely decreased lifespan than that exposed to Ca, or Ca+Fe. Moreover, the baseline toxicity on lifespan was doubled by doubling the concentration of combined metals (Ca+Al+Fe) in spiking test in original effluent (oe), and lifespan defects in oe+Ca+Al+Fe exposed nematodes were more severe than that in Ca+Al+Fe exposed nematode. Therefore, Ca+Al+Fe exposure may largely explain the formation of decreased lifespan induced by the examined industrial effluent. Furthermore, the observed reduction of lifespan induced by the combination of high level of Ca with other metals may be at least partially independent of the insulin-like pathway.

Key words: lifespan; calcium; combinational toxicity; toxicity identification evaluation (TIE); *Caenorhabditis elegans*

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Introduction

It has been shown that bioassay and chemical analysis can be effectively combined to identify and determine the degrees of pollution in natural waters and soils (Kwok et al., 2005). Toxicity identification evaluation (TIE) is a bioassay-directed fractionation protocol first developed by USEPA (1991) to determine what fractions of chemicals causing the observed adverse effects on the bioassay and to allow the isolation of compounds in a complex mixture. This method has been used in characterizing and identifying toxicants in samples of freshwater and marine effluents, receiving waters, interstitial waters, sediment pore waters, and whole sediments (Ankley et al., 1992; US EPA, 1996; Burgess et al., 2000; Ho et al., 2002; Fjällborg et al., 2006). In phase II of TIE, the individual toxicant(s) can be isolated and tentatively identified with the aid of analytical techniques and toxicity evaluation (Hogan et al., 2005). Furthermore, in the final TIE phase, whether the suspected toxicant is in fact the true toxicant would be directly determined (Mount and Norberg-King, 1993).

Nematode *Caenorhabditis elegans* is a free-living soil nematode that has been extensively used as a model organism in the study of genetics and developmental biology (Riddle et al., 1997). *C. elegans* is sensitive to some testing toxicants, easy to culture and manage in the laboratory, and available throughout the year, and it has been developed for ecological risk assessment in soil and water by virtue of these properties (Power and de Pomerai, 1999; Peredney and Williams, 2000; Ura et al., 2002; Wang et al., 2008; Li et al., 2009). A standardized method for conducting laboratory soil toxicity tests using *C. elegans* was published in the America Society for Testing and Materials Guide E2172-01 (2002). Moreover, several endpoints, such as mortality, lifespan, reproduction, behavior, and body length, have been used for acute toxicity testing with *C. elegans* (Mutwakil et al., 1997; Power and de Pomerai, 1999; Dhawan et al., 1999; Peredney and Williams, 2000; Ura et al., 2002; Chu and Chow, 2002; Swain et al., 2004; Wang and Xing, 2008; Du and Wang, 2009; Xing et al., 2009). Among these endpoints, lifespan has been explored to evaluate the metal or detergent toxicity (Lin et al., 2006; Harada et al., 2007; Wang et al., 2007a, 2007b; Wang and Wang, 2008a, 2008b), and to study the metallothionein function in nematodes (Swain et al., 2004; Hughes and Stürzenbaum, 2007).

The TIE method has been successfully used in the toxicity evaluation in a paper recycling mill effluent by coupling bioindicator of *C. elegans* (Wang et al., 2008). In the examined industrial effluent, Wang et al. (2008) found

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that only the toxicities from mixed cellulose filtration and EDTA treatment were similar to the baseline aging toxicity, suggesting the suspect toxicants inducing aging toxicity might largely be the metal substances in this industrial effluent. Moreover, high levels of Ca (1069 μg/mL), Al (33.4 μg/mL), and Fe (43.1 μg/mL) in this effluent might account for the severely observed aging toxicity on exposed nematodes (Wang et al., 2008). Therefore, the first aim of this project was to investigate the possible combinational toxicity effects of high level of Ca with metals of Fe and Al at assayed concentrations in the paper recycling mill effluent on lifespan. In addition, considering the fact that insulin signaling is the central molecular mechanism controlling the nematode aging (Murphy et al., 2003), we also examined the effects of insulin signaling on the possible combinational effects of Ca with metals of Fe and Al in the examined industrial effluent in inducing the decreased lifespan.

1 Materials and methods

1.1 Sample collection

Water sample was collected from the effluent of a paper recycling mill in Nanjing as described in our previous work (Wang et al., 2008). Upon the arrival of the water sample, the sample was stored at 4°C until required.

1.2 Reagents

The metal concentrations for Ca, Fe, and Al used in this study were based on the analysis of the paper recycling mill effluent as the previously described work (Wang et al., 2008). The metal concentrations were Ca (1069 μg/mL), Fe (43.1 μg/mL), and Al (33.4 μg/mL), respectively. The corresponding used reagents were $CaCl_2$, $FeSO_4$ and $AlCl_3$, respectively. Metal concentrations of exposed solutions were analyzed by atomic absorption spectrophotometry (AAS; Pye-Unicam model SP9, Cambridge, UK). All the chemicals were obtained from Sigma-Aldrich (St. Louis, USA).

1.3 Preparation of nematode cultures

Nematodes used in the present study were wild-type N2, and long-lived mutant of *daf-2(e1370)*, originally obtained from the *Caenorhabditis* Genetics Center (funded by the NIH National Center for Research Resource, USA). In *C. elegans*, the lifespan is regulated hormonally by an insulin/IGF-like signaling pathway. In DAF-2 (insulin receptor-like protein) pathway mutants, DAF-16, a forkhead-family transcription factor, accumulates in the nuclei of many cell types, where it results in changes in the expression of a wide variety of response, and thereby extends lifespan (Murphy et al., 2003). The strains were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20°C as described by Brenner (1974). Gravid nematodes were washed off the plates into centrifuge tubes, and were lysed with a bleaching mixture (0.45 mol/L NaOH, 2% HOCl). Age synchronous populations of nematodes (L4-stage larvae) were obtained by the collection as described by Donkin and Williams (1995). The examined nematodes were washed with double-distilled water twice, followed by washing with modified K medium once (50 mmol/L NaCl, 30 mmol/L KCl, 10 mmol/L NaOAc, pH 5.5) (Williams and Dusenbery, 1990). Exposures were performed in 12-well sterile tissue culture plates as described by Mutwakil et al. (1997). All exposures were 2-day long and were carried out in 20°C incubator in the presence of food.

1.4 Life span assay

The method was performed as previously described in literature (Shen et al., 2007; Wang and Wang, 2008a). Before the lifespan assay, the metal exposed nematodes were transferred into new NGM plates seeded with *E. coli* OP50, and stayed for 1 hr, then transferred into another NGM plates for lifespan assay. In this test, the metal exposed and control hermaphrodite adults were transferred into new NGM plates daily from day 1 to day 4. From day 5, the examined nematodes would be not transferred into new NGM plates again. The nematodes were checked every 2 days from day 1, and were scored as dead when they did not move even after repeated taps with a pick. The number of survival nematodes was counted every 2 days. During the assay, the nematodes were cultured in 20°C incubator in the presence of food. Approximately 50 animals were selected and used for each lifespan trial. For life span, graphs are representative of three trials.

1.5 Dauer formation assay

The dauer larva, an alternative third larval (L3) form usually induced by lack of food, high temperature, and high concentration of dauer pheromone, is long-lived reproductive immature, and resistant to desiccation and starvation (Inoue and Thomas, 2000). In the starvation assay, dauer formation is induced by allowing plates of nematodes to starve naturally, which causes a very strong dauer induction (Inoue and Thomas, 2000). The starvation assay method was basically performed following literature (Inoue and Thomas, 2000; Shen et al., 2007). Before the lifespan assay, the metal exposed nematodes were transferred into new NGM plates seeded with *E. coli* OP50, and stayed for 1 hr, and then transferred into another NGM plates for lifespan assay. About four exposed and control pregnant adult nematodes per plate were allowed to lay eggs on NGM plates for 4–6 hr at 20°C. These progeny were then shifted to 27°C, and 72 hours later, the number of dauer or adult nematodes was counted. In order to distinguish the adult and dauer nematodes, while the food on NGM plates was almost exhausted, the plates were flooded with 1% sodium dodecyl sulfate (SDS) solution. The adult nematodes would be dead, and they did not move even after repeated taps with a pick. In contrast, the dauer nematodes were resistant to SDS solution, and could move even after repeated taps with a pick.

1.6 Statistical analysis

All data in this article were expressed as means ± SD. Graphs were generated using Microsoft Excel (Microsoft

Corp., Redmond, USA). Multi-factor analysis of variance (ANOVA) followed by a Dunnett's *t*-test was used to determine the significance of the differences in mean lifespans or dauer formation. The probability levels of 0.05 (*t*-test at 95% confidence level) and 0.01 (*t*-test at 99% confidence level) were considered statistically significant.

2 Results

2.1 Effects of single or combined metal exposure on the lifespan in nematodes

We first examined the possible combined metal toxicity (Ca + Al, Ca + Fe, Al + Fe, or Ca + Al + Fe) on nematode lifespan. The metal concentrations were prepared according to the identification in the paper recycling mill effluent (Wang et al., 2008). As shown in Fig. 1a, exposure to Al + Ca and Ca + Fe caused more severely decreased maximum lifespan than that in nematodes exposed to single Ca, Al or Fe alone; however, exposure to Fe + Al did not increase the maximum lifespan compared to that exposed to Fe or Al alone. Moreover, the lifespan defect in Ca + Al exposed nematodes was more severe than that in Ca ($P < 0.01$) or Al ($P < 0.01$) exposed nematodes (Fig. 1b, Table 1). Similarly, the lifespan defect in Ca + Fe exposed nematodes was more severe than that in Ca ($P < 0.01$) or Fe ($P < 0.05$) exposed nematodes (Fig. 1c, Table 1). In contrast, the combined effect of Al + Fe on mean lifespan of exposed nematodes was neutralizing (Fig. 1d, Table 1). Moreover, exposure to Ca + Al + Fe led to more severely decreased lifespan than that in nematodes exposed to Ca ($P < 0.01$), or Ca + Fe ($P < 0.01$) (Fig. 1e). The induced lifespan toxicity from Ca + Al was similar to that in Ca + Al + Fe exposed nematodes (Fig. 1e). In addition, the identified high level of Ca had only a neutralizing effect on other trace metals in inducing lifespan toxicity in the assayed industrial effluent, such as Mg (37.2 μg/mL), Mn (0.5 μg/mL), Zn (1.46 μg/mL), Cd (< 0.001 μg/mL), Co (< 0.001 μg/mL), Cr (< 0.01 μg/mL), Ni (< 0.01 μg/mL), and Pb (< 0.001 μg/mL) (data not shown).

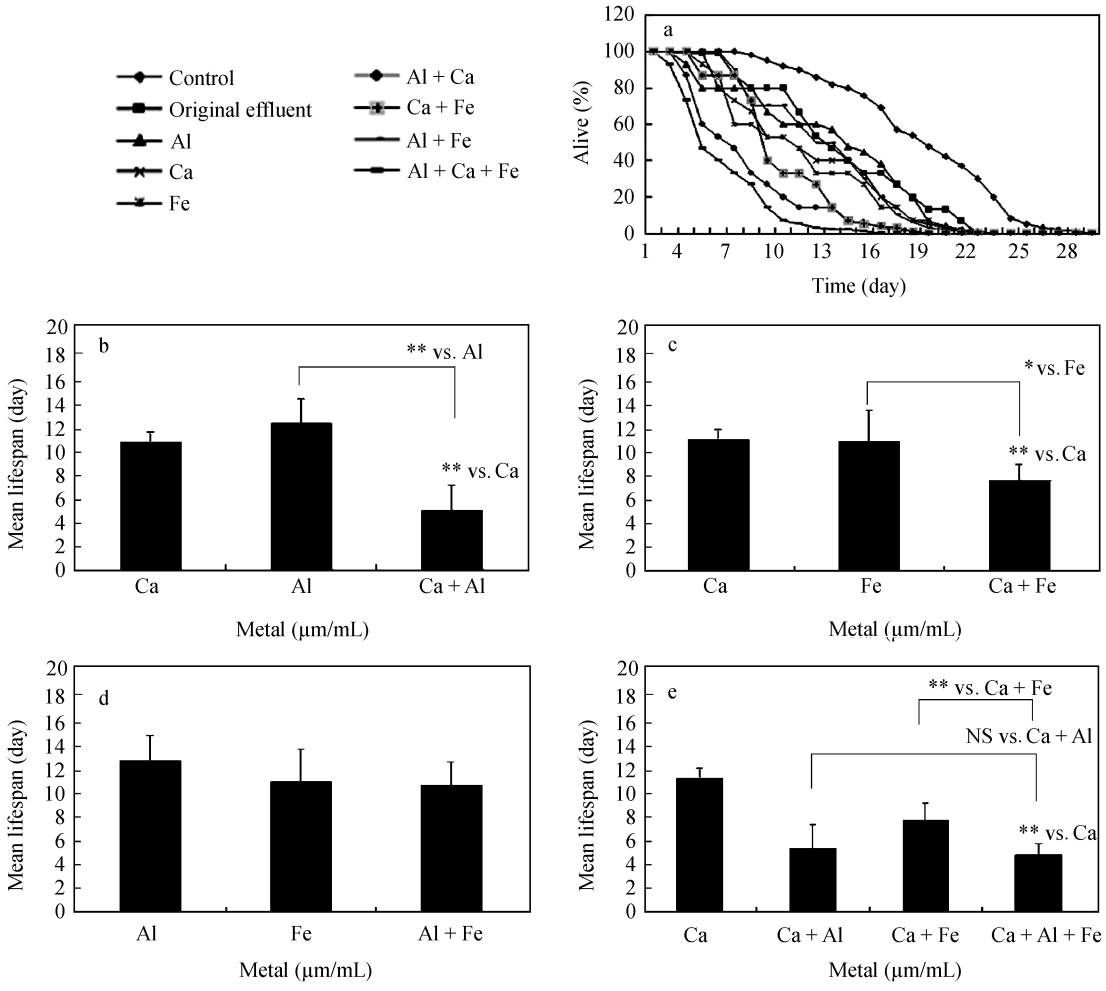

Fig. 1 Life spans in wild-type N2 nematodes exposed to single or combined metals. (a) effects of single or combined metal exposure on nematode lifespans; (b) synergistic effect was observed between Ca and Al in inducing lifespan toxicity; (c) synergistic effect was observed between Ca and Fe in inducing lifespan toxicity; (d) no synergistic effect could be detected between Al and Fe in inducing lifespan toxicity; (e) effect of triple metal exposure (Ca+Al+Fe) on nematode lifespans. Metal concentrations used were 33.4 μg/mL (Al), 1069 μg/mL (Ca), and 43.1 μg/mL (Fe), respectively. Control, wild-type N2 nematode. Bars represent means ± SD. * $P < 0.05$, ** $P < 0.01$.

Table 1 Mean lifespan caused by various metal toxicity in *C. elegans*

Phase extraction	Mean lifespan (day)	Ratio vs. oe[a]	Ratio vs. con[b]	Maximum lifespan (day)[c], *	*N*	*P* vs. oe[d]	*P* vs. con[e]
Control	14.8 ± 0.8			26, 32	47		
oe	10.7 ± 0.2		0.72	19, 18	51		<0.01
Al	12.5 ± 2.1	1.17	0.85	22, 24	49	NS	NS
Ca	10.9 ± 0.9	1.02	0.74	22, 22	51	NS	<0.05
Fe	10.7 ± 2.7	1.00	0.72	22, 21	51	NS	<0.05
Al+Ca	5.1 ± 2.1	0.48	0.35	18, 21	54	<0.01	<0.01
Fe+Ca	7.5 ± 1.4	0.71	0.51	18, 20	47	<0.01	<0.01
Al+Fe	10.5 ± 1.9	0.98	0.71	21, 19	51	NS	<0.01
Al+Ca+Fe	4.7 ± 0.9	0.44	0.32	15, 17	47	<0.01	<0.01
daf-2	53.5 ± 5.1	5.00	3.61	69, 74	52	<0.01	<0.01
daf-2+Al	19.5 ± 2.1	1.82	1.32	31, 28	46	<0.01	<0.01
daf-2+Ca	18.5 ± 1.9	1.73	1.25	30, 32	49	<0.01	<0.05
daf-2+Fe	19.0 ± 3.1	1.78	1.28	32, 33	51	<0.01	NS
daf-2+Ca+Fe	8.5 ± 1.9	0.79	0.57	17, 19	49	<0.01	<0.01
daf-2+Al+Ca	8.6 ± 2.7	0.80	0.58	13, 11	47	<0.01	<0.01
daf-2+Al+Fe	11.5 ± 1.7	1.08	0.78	28, 25	52	<0.05	<0.05
daf-2+Al+Ca+Fe	6.5 ± 2.2	0.61	0.44	11, 12	47	<0.01	<0.01
oe+Al+Ca+Fe	4.0 ± 3.7	0.37	0.27	12, 9	51	<0.01	<0.01

Results of one typical experiment are shown; NS means no significant differences.
con: control; oe: original effluent.
Metal concentrations used in this assay were 33.4 μg/mL (Al), 1069 μg/mL (Ca), and 43.1 μg/mL (Fe), respectively.
[a] Ratio of mean life span divided by that of original effluent; [b] ratio of mean life span divided by control; [c] the average maximum lifespans of three trials; [d] probability of survival being different from that of original effluent; [e] probability of survival being different from control.
* The values represent the maximum and the minimum values of maximum lifespan, respectively.

2.2 Combination of Ca + Al + Fe explains the major lifespan toxicity in exposed nematodes

The aim of the phase III in TIE method is to determine or confirm whether the suspected toxicant is in fact the true toxicant (Mount and Norberg-King, 1993). For the spiking test in original effluent, the results showed that the baseline toxicity on both maximum lifespan and mean lifespan could be severely strengthened by doubling the concentration of combined metals (oe + Ca + Al + Fe) (Fig. 2, Table 1). In addition, the defects of maximum and mean lifespan in oe + Ca + Al + Fe exposed nematodes were more severe than those in Ca + Al + Fe exposed nematodes (Fig. 2, Table 1). Therefore, the results obtained in the current work suggest that the combined metals (Ca + Al + Fe) were the major toxicants for lifespan toxicity observed in the examined industrial effluent.

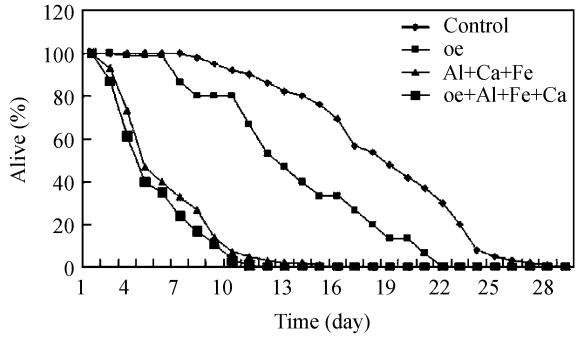

Fig. 2 Life spans of the phase III spiking test for original effluent (oe) in wild-type N2 nematodes. Metal concentrations used were 33.4 μg/mL (Al), 1069 μg/mL (Ca), and 43.1 μg/mL (Fe), respectively. Control: wild-type N2 nematode.

2.3 Effects of single or combined metal exposure on the lifespan in *daf-2* mutant nematode

Furthermore, we investigated the possible effects of single and combined metal exposure on lifespan in *daf-2* mutant nematodes. As shown in Fig. 3 and Table 1, exposure to single metal (Ca, Al or Fe) could obviously lengthen the lifespan of *daf-2* mutants. The mean lifespans of *daf-2* mutant nematodes exposed to single metal (Ca, Al or Fe) were nearly close to that of wild-type N2. Moreover, exposure to combined metals (Ca + Al, Ca + Fe, Al + Fe, Ca + Al + Fe) resulted in more severe lifespan defects than those exposed to single metals (Ca, Al, or Fe) in *daf-2* mutant nematodes. The mean lifespans of *daf-2* mutant nematodes exposed to combined metals were much shorter than the mean lifespan of wild-type N2.

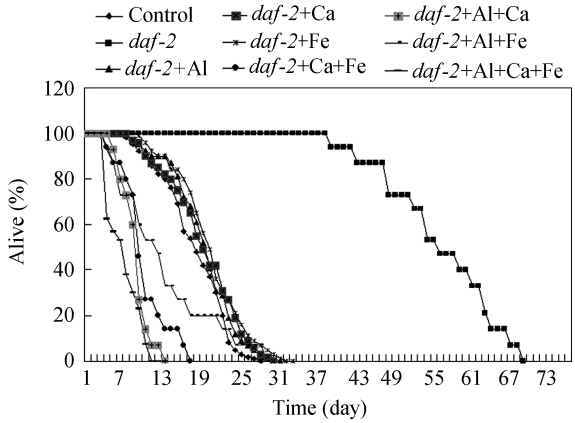

Fig. 3 Life spans in *daf-2* mutant exposed to single or combined metals. Metal concentrations used were 33.4 μg/mL (Al), 1069 μg/mL (Ca), and 43.1 μg/mL (Fe), respectively. Control: wild-type N2 nematode.

2.4 Effects of single or combined metal exposure on dauer formation in wild-type N2 and *daf-2* mutant nematodes

As shown in Fig. 4, no obvious differences of dauer formation could be detected in single or combined metal exposed wild-type N2 and *daf-2* mutant nematodes from that in control.

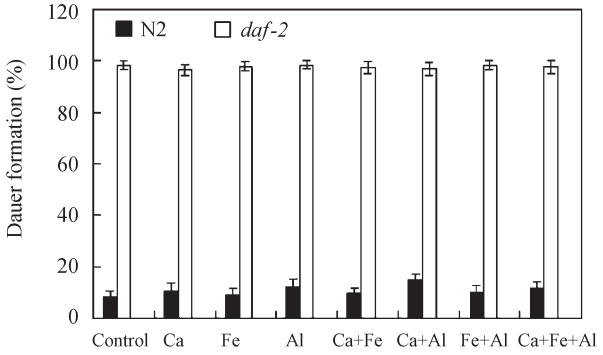

Fig. 4 Effects of single or combined metal exposure on dauer formation in wild-type N2 and *daf-2* mutant nematodes. Metal concentrations used were 33.4 μg/mL (Al), 1069 μg/mL (Ca), and 43.1 μg/mL (Fe), respectively. Control: without metal exposure. Bars represent means ± SD.

3 Discussion

In the present study, we investigated the possible interaction of identified toxic metals (Ca, Al, and Fe) in a paper recycling mill effluent in inducing the decreased lifespan in nematodes. Our result suggest that exposure to Ca + Al caused more severe reduction of lifespan than that exposed to Ca, or Al, and exposure to Ca + Fe resulted in more severe reduction of lifespan than that exposed to Ca, or Fe. In addition, exposure to Ca + Al + Fe led to more severe reduction of lifespan than that exposed to Ca, or Ca + Fe, and the decreased lifespan induced by Ca + Al was similar to that in Ca + Al + Fe exposed nematodes. Therefore, the data provided in this project raise such a notion that exposure to the mixture of high level of Ca with some other specific metals, such as Fe and Al, may induce more severe toxicity than that exposure to single metal solution. Effects of the combination of Ca with other metals have also been reported in other organisms. For example, effects of Ca and Mg ratios on the toxicity of Cu to five aquatic species had been examined in freshwater, and Cu toxicity increased at higher Ca:Mg ratios for *Daphnia magna* (Naddy et al., 2002). The importance of Ca was also reported in modifying the acute toxicity of sodium sulphate to *Hyalella azteca* and *D. magna* using exposure water with different levels of water hardness and Ca-Mg ratios (Davies and Hall, 2007). Moreover, the influence of Ca, humic acid and pH on Pb accumulation and toxicity in the fathead minnow during prolonged water-borne Pb exposure suggest that water chemistry characteristics like Ca and dissolved organic carbon should be considered for chronic water quality criteria (Grosell et al., 2006).

In this study, our result also demonstrated that the baseline toxicity on lifespan could be severely strengthened by doubling the concentration of combined metals (oe + Ca + Al + Fe), and lifespan defects in oe + Ca + Al + Fe exposed nematodes were more severe than those in Ca + Al + Fe exposed nematodes, suggesting that the combined metals (Ca + Al + Fe) were the major toxicants for inducing the decreased lifespan in the examined paper recycling mill effluent. Moreover, the data obtained here also suggest that some unknown substances might exist in this industrial effluent, which could counteract part of the toxicity from the combination of high level of Ca with metals of Al and Fe. The exposure to fractions of EDTA manipulations from the examined effluent could not result in severe defects of reproduction and locomotion behaviors of exposed nematodes (Wang et al., 2008), which further suggest the possible existence of this kind of substances in the examined industrial effluent.

In addition, our result suggest that exposure to combined metals (Ca + Al, Ca + Fe, Al + Fe, Ca + Al + Fe) resulted in more severe lifespan defects than those exposed to single metals (Ca, Al, or Fe) in *daf-2* mutant nematodes, and the mean lifespans of *daf-2* mutant nematodes exposed to single metal (Ca, Al or Fe) were nearly close to wild-type. It has been reported that *daf-2* mutation resulted in increased Cd^{2+} and Cu^{2+} resistance in a 24-hr lethal toxicity assay, and metallothionein 1 (MT1) mRNA levels were significantly higher in *daf-2* mutants compared to wild-type *C. elegans* under basal conditions (Barsyte et al., 2001). Therefore, the observed increased lifespan toxicity from the combination of high level of Ca with other metals may be at least partially independent of the insulin-like pathway. Moreover, no obvious differences of dauer formation could be observed in single or combined metal exposed wild-type N2 and *daf-2* mutant nematodes from that in control, suggesting that the observed increased lifespan toxicity for Ca with metals of Al or Fe may be also independent of the dauer pathway in exposed nematodes. Thus, specific mechanism(s) may activate the increased toxicity from the combination of high level of Ca with other metals.

4 Conclusions

The Ca + Al + Fe exposure might explain most of the toxicity on lifespan from the examined paper recycling mill effluent, and Ca can may exert at least partially synergistic effects on the Al or Fe toxicity during the decreased lifespan in nematodes. Especially, the combination of high level of Ca with other metals plays a pivotal role in inducing the formation of decreased lifespan in nematodes exposed to the examined industrial effluent.

Acknowledgments

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