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Behavior toxicity to *Caenorhabditis elegans* transferred to the progeny after exposure to sulfamethoxazole at environmentally relevant concentrations

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Abstract

Sulfamethoxazole (SMX) is one of the most common detected antibiotics in the environment. In order to study whether SMX can affect behavior and growth and whether these effects could be transferred to the progeny, *Caenorhabditis elegans* was exposed at environmentally relevant concentrations for 24, 48, 72 and 96 hr, respectively. After exposure, the exposed parent generation (P_0) was measured for behavior and growth indicators, which were presented as percentage of controls (POC). Then their corresponding unexposed progeny (F_1) was separated and measured for the same indicators. The lowest POC for P_0 after 96 hr-exposure at 100 mg/L were 37.8%, 12.7%, 45.8% and 70.1% for body bending frequency (BBF), reversal movement (RM), Omega turns (OT) and body length (BL), respectively. And F_1 suffered defects with the lowest POC as 55.8%, 24.1%, 48.5% and 60.7% for BBF, RM, OT and BL, respectively. Defects in both P_0 and F_1 showed a time- and concentration-dependent fashion and behavior indicators showed better sensitivity than growth indicator. The observed effects on F_1 demonstrated the transferable properties of SMX. Defects of SMX at environmental concentrations suggested that it is necessary to perform further systematical studies on its ecological risk in actual conditions.

Key words: sulfamethoxazole; transferable property; *Caenorhabditis elegans*; environmental concentration

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Introduction

The occurrence of antibiotics in the environment, and their potential biological effects, make them an emerging threat to the ecological stability (Hernando et al., 2006; Santos et al., 2010). Sulfamethoxazole (SMX) is among the most common existing antibiotics in the environment with the detected frequency up to 73% (Watkinson et al., 2009) and its environmental concentrations are mainly at the magnitude of ng/L to low μg/L (Chang et al., 2010; García-Galán et al., 2010; Shelver et al., 2010). Although the environmental concentrations are at trace levels, SMX is still gaining more and more ecological concern for its pseudopersistance caused by the continuous introduction in the environment (Hernando et al., 2006).

To verify the potential risk of SMX, toxicity effects have been studied on various organisms including luminescent bacteria, algae, invertebrates and fish (Park and Choi, 2008). Most of these studies focused on acute toxicities, and corresponding median lethal concentration (LC_{50}) or median effective concentration (EC_{50}) values for SMX were reported in the level of mg/L, e.g., 25.2 mg/L for the $EC_{50,\,24\,hr}$ to *D. magna* (Isidori et al., 2005). On the other hand, the chronic toxicity data for SMX are available for only a few organisms. For example, *L. gibba* suffered toxic defects with 249 μg/L and 81 μg/L as the $EC_{50,\,7\,days}$ of frond number and wet mass (Brain *et al.*, 2004). However, employed concentrations and reported LC or EC values of SMX in current toxicity studies were generally much higher than its environmental levels, and the long-term ecological risk of SMX in the actual environment remains poorly studied. In addition, the confirmed bacterial resistance to SMX in the environment (Chelossi et al., 2003; Le et al., 2005) demonstrated its potential to affect the offspring of organisms, and put more significance in studying the transferable properties of SMX at environmentally relevant concentrations.

Caenorhabditis elegans was chosen to facilitate the research on the transferable properties. This free-living nematode was employed in ecological researches due to its short but precise life cycle and multiple sensitive endpoints (Anderson et al., 2001). Multiple sublethal endpoints including behavior and growth indicators were successfully used in demonstrating the transferable properties of heavy metals (Wang et al., 2007; Wang and Wang, 2008). Thus,

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C. elegans was proved appropriate for the test organism.

In this study, *C. elegans* was exposed to SMX at environmentally relevant concentrations to study whether SMX can affect behavior and growth, and whether these effects could be transferred from the exposed parent generation to the unexposed progeny. Body bending frequency (BBF), reversal movement (RM) and Omega turns (OT) were chosen to indicate behavior effects, while body length (BL) was chosen to represent the growth effect.

1 Materials and methods

1.1 Tested chemical

Sulfamethoxazole (CAS: 723-46-6, $C_{10}H_{11}N_3O_3S$), was obtained from Sigma-Aldrich (USA). SMX stocking solution with highest concentration as 200 mg/L was pre-dissolved in sterilized distilled water containing 1% dimethyl sulphoxide (DMSO, *V*/*V*) (Mukai et al., 2008), and maintained in the dark at 4°C. The DMSO (CAS: 67-68-5) was obtained from Sinopharm Chemical Reagent Co. Ltd., Shanghai, China.

1.2 Preparation of nematode cultures

All nematodes used were *C. elegans*, wild-type N2. They were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20°C as described by previous study (Brenner, 1974).

For age-synchronization, gravid nematodes were gently shaken at room temperature in fresh clorox solution containing 1% NaClO (4%–6%)/0.5 mol/L NaOH (Emmons et al., 1979). After 10–15 min, eggs, which are resistant to this treatment, were isolated and washed for several times. Eggs were then placed onto NGM plates with *E. coli* OP50 lawn, and incubated for 36 hr to obtain the age-synchronized L4 nematodes for toxicity tests. The reason for using L4 stage was that the sperm, ovum and eggs start to form around L4-adult molt (Hill et al., 2006), and the progeny of the nematode can be affected by exposure lasting long enough to cover the whole processes.

1.3 Toxicity experiment design

The steps for the toxicity test are shown in Fig. 1. Ten different concentration groups of SMX were diluted from the stocking solution with sterilized distilled water containing 1% DMSO and then arranged in 96-well sterile culture plates with eight wells as parallel for each group. Each test well typically contained 200 μL mixture of two hundred nematodes and SMX. The final nominal concentrations for exposure of SMX were 100 mg/L, 10 mg/L, 1 mg/L, 100 μg/L, 10 μg/L, 1 μg/L, 100 ng/L, 10 ng/L, 1 ng/L and 0 (control). DMSO concentration was 0.5% (*V*/*V*) for all groups including the control as previous studies (Bhaiya et al., 2006; Dengg and van Meel, 2004). Four identical 96-well plates were used for exposure time 24, 48, 72 and 96 hr, respectively and all experiments were carried out at 20°C in the absence of food. After exposure, nematodes in three wells for each of the ten concentration groups were used for the indicator measurements of the exposed generation marked as P_0.

In order to measure the indicators of the unexposed progeny (marked as F_1), five remaining wells for each of the ten concentration groups were transferred to ten sets of SMX-free NGM plates with *E. coli* OP50 as food. The nematodes were allowed to grow for 36 hr to produce enough eggs for age-synchronization as described earlier, after which the second age-synchronized eggs were allowed to grow for 36 hr. Then nematodes were captured for the indicator measurements of F_1.

The dissecting microscope used to capture the images and videos was XTL-3400C, which was obtained from Caikon Optical Instrument Co., Ltd., Shanghai, China.

1.4 Growth indicator

The effects on growth were determined according to the method of Anderson et al. (2001) with a little modification. Nematodes were transferred onto SMX-free NGM plates without *E. coli* OP50 as food after washed with sterilized

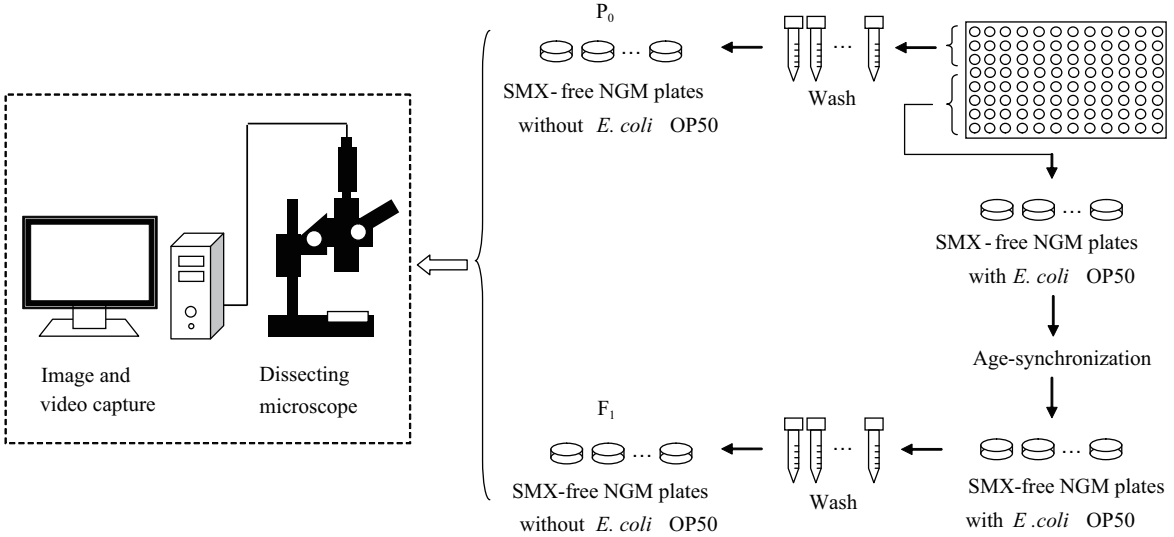

Fig. 1 Diagram of the steps for the toxicity test with *C. elegans*. 96-well sterile plates were used with each well typically containing 200 μL mixture of nematodes and SMX of different concentrations. Every concentration had eight wells as parallel and different concentration groups used separate NGM plates for exposed parent generation (P_0) or unexposed progeny (F_1).

distilled water. After 12 hr allowing water evaporate, the nematodes were captured with the dissecting microscope. A sequence of polylines was drawn following the nematodes' midline from the head to the tail and back to the head. The body length was calculated as BL = (length of the polylines)/2. At least twenty nematodes were repeated for each treatment.

1.5 Behavior indicators

Behavior indicators were performed as previously described for *C. elegans* (Wang and Wang, 2008; Wang and Xing, 2008) with some modifications. After the image capture for growth indicator, nematodes were immediately captured for the videos. Then nematodes were scored for the number of BBF, RM and OT in an interval of 60 sec. BBF was the times when the posterior bulb of the pharynx changed the direction along the vertical direction of the traveling path within 60 sec. RM was counted as every time when the traveling direction changed including backward turns and OT. OT was referred as when the head of the nematode touched its tail looking like the shape of the Greek letter Omega. At least six animals were examined for each treatment.

1.6 Statistical analysis

Inhibition rates for growth (BL) and behavior indicators (BBF, RM and OT) were all calculated as a percentage of controls (POC) as in previous literature (Anderson et al., 2001). All data were checked for normality using Anderson-Darling normality tests. One-way ANOVA (using Tukey test) and linear regression were all calculated with the Origin Pro 7.5 (Origin Lab Corp., USA), and the probability levels of 0.05 and 0.01 were considered statistically significant. For one-way ANOVA, data for different exposure time were all compared with 24 hr-exposure time for the same indicator.

2 Results

2.1 Behavior defects caused by sulfamethoxazole

Behavior defects of P_0, which were exposed to SMX directly, were indicated by BBF, RM and OT, and results are shown in Fig. 2 and Table 1, Fig. 3 and Table 2, and Fig. 4 and Table 3, respectively. The same indicators of F_1, which were the offspring of P_0 and were not exposed to SMX, are also shown for the convenience of comparison.

Results in Fig. 2 and Table 1, Fig. 3 and Table 2, and Fig. 4 and Table 3, showed that after exposure to SMX,

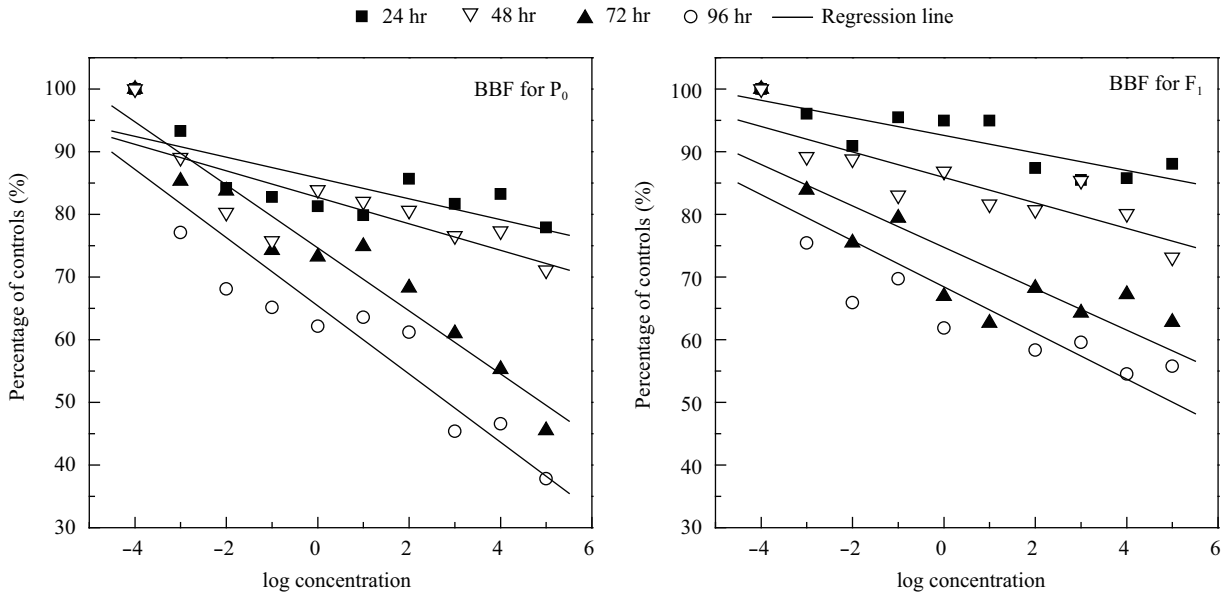

Fig. 2 Concentration-response relationship of body bending frequency (BBF) for P_0 and F_1. P_0: exposed parent generation; F_1: unexposed progeny of P_0. Concentration unit was μg/L before logarithm.

Table 1 Fitted linear functions and ECs of BBF for P_0 and F_1

	Exposed time (hr)	Functions	R^2	EC_{10} (μg/L)	EC_{15} (μg/L)	EC_{20} (μg/L)	EC_{25} (μg/L)	EC_{50} (μg/L)
P_0	24	$y = -1.66x + 85.81$	0.5646	2.99E-03	3.08	3.16E+03	>1.00E+05	>1.00E+05
	48	$y = -2.11x + 82.72$	0.6221	3.55E-04	8.31E-02	1.95E+01	4.56E+03	>1.00E+05
	72	$y = -5.03x + 74.68$	0.9377*	9.00E-04	8.88E-02	8.76E-02	8.64E-01	8.06E+04
	96	$y = -5.44x + 65.42$	0.8664**	<1.00E-04	2.52E-04	2.09E-03	1.73E-02	6.83E+02
F_1	24	$y = -1.40x + 92.61$	0.7041	7.32E+01	>1.00E+05	>1.00E+05	>1.00E+05	>1.00E+05
	48	$y = -2.03x + 85.90$	0.7399*	9.56E-03	2.78	8.06E+02	>1.00E+05	>1.00E+05
	72	$y = -3.30x + 74.75$	0.7072**	<1.00E-04	7.83E-04	2.57E-02	8.40E-01	>1.00E+05
	96	$y = -3.68x + 68.43$	0.6935**	<1.00E-04	<1.00E-04	7.18E-04	1.64E-02	>1.00E+05

ECs: effective concentrations.
* $P < 0.05$ vs. 24 hr-exposure, ** $P < 0.01$ vs. 24 hr-exposure.

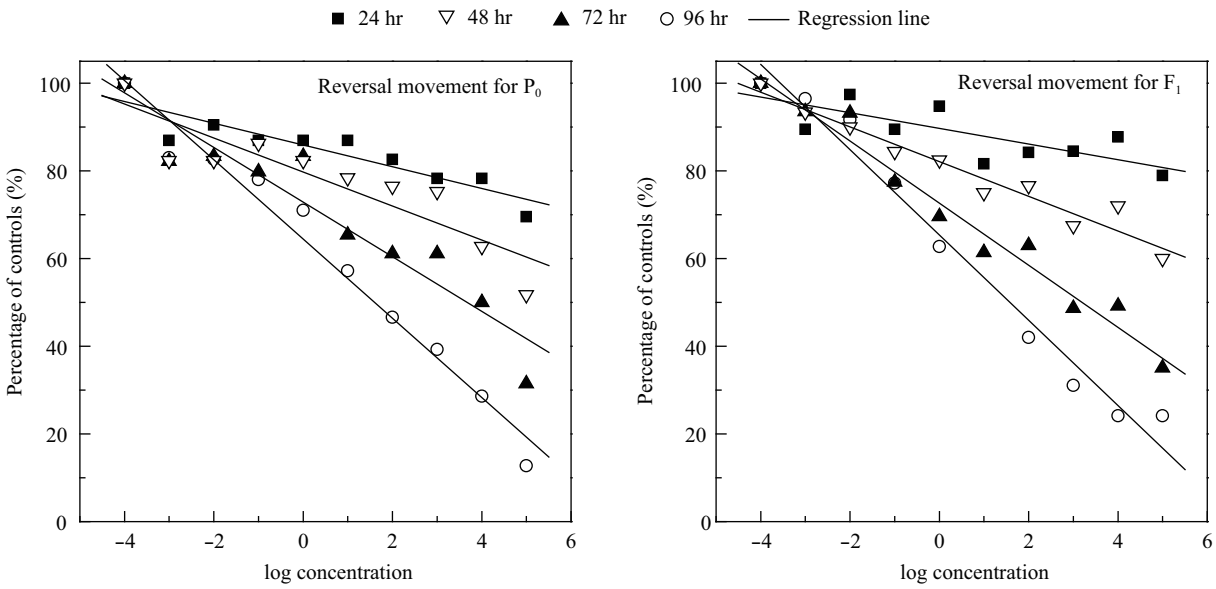

Fig. 3 Concentration-response relationship of reversal movement for P_0 and F_1. Concentration unit was μg/L before logarithm.

Table 2 Fitted linear functions and ECs of reversal movement for P_0 and F_1

	Exposed time (hr)	Functions	R^2	EC_{10} (μg/L)	EC_{15} (μg/L)	EC_{20} (μg/L)	EC_{25} (μg/L)	EC_{50} (μg/L)
P_0	24	$y = -2.48x + 85.93$	0.8384	2.28E-02	2.37	2.46E+02	2.55E+04	>1.00E+05
	48	$y = -3.88x + 79.74$	0.8061	2.25E-02	1.43E-01	8.57E-01	1.67E+01	>1.00E+05
	72	$y = -6.23x + 72.90$	0.8975*	1.80E-03	1.14E-02	7.25E-02	4.60E-01	4.74E+03
	96	$y = -9.04x + 64.43$	0.9730*	1.48E-03	5.30E-03	1.90E-02	6.77E-02	3.95E+01
F_1	24	$y = -1.79x + 89.69$	0.621	6.71E-01	4.17E+02	>1.00E+05	>1.00E+05	>1.00E+05
	48	$y = -3.96x + 82.12$	0.9493	1.02E-02	1.87E-01	3.43	6.28E+01	>1.00E+05
	72	$y = -7.09x + 72.64$	0.9771*	3.56E-03	1.81E-02	9.16E-02	4.65E-01	1.56E+03
	96	$y = -9.73x + 65.36$	0.9592*	2.94E-03	9.58E-03	3.13E-02	1.02E-01	3.79E+01

* $P < 0.05$ vs. 24 hr-exposure, ** $P < 0.01$ vs. 24 hr-exposure.

the parent generation of nematodes (P_0) suffered behavior effects with the lowest POC as 37.8% (EC_{50} = 6.83E+02 μg/L), 12.7% (EC_{50} = 3.95E+01 μg/L) and 45.8% (EC_{50} = 2.77E+03 μg/L) after 96 hr-exposure at highest exposure concentration (100 mg/L) for BBF, RM and OT, respectively. The results above also showed that the progeny (F_1) suffered defects even without direct exposure to SMX, and the lowest POC for F_1, whose parent generation were exposed for 96 hr-exposure at 100 mg/L, as 55.8% (EC_{50} > 1.00E+05 μg/L), 24.1% (EC_{50} = 3.79E+01 μg/L), and 48.5% (EC_{50} = 1.39E+04 μg/L) for BBF, RM and OT, respectively.

For P_0, many EC values were under environmental concentration levels as shown in Tables 1, 2 and 3. Effects for all three behavior indicators had a time- and concentration-dependent fashion, for POC decreased with longer exposure time and higher concentration of SMX. And for the same indicator and same generation, statistical significance increased with longer exposure time (Tables 1, 2 and 3).

2.2 Growth defects caused by sulfamethoxazole

Growth defects of P_0 indicated by BL are shown in Fig. 5. Results in Fig. 5 and Table 4 showed that after exposure to SMX, P_0 suffered growth defects with the lowest POC as 70.1% (EC_{50} > 1.00E+05 μg/L) after 96 hr-exposure at 100 mg/L. And F_1 also suffered defects with the lowest POC as 60.7% (EC_{50} > 1.00E+05 μg/L) for the progeny of those which were exposed for 96 hr-exposure at highest exposure concentration. Moreover, EC_{10} and EC_{15} values of growth indicator for F_1 were under environmental level. The effect on growth had a time- and concentration-dependent fashion, for POC decreased with longer exposure time and higher concentration of SMX. And for the same generation, statistical significance increased with longer exposure time (Table 4).

3 Discussion

Sulfamethoxazole resulted in behavior and growth defects on exposed nematodes in a time- and concentration-dependent way. Behavior indicators showed consistent results with previous related studies. The EC_{50} values for behavior indicators were similar with previous study on *L. gibba* with multiple endpoints showing sublethal effects (Brain et al., 2004). The behavior defects observed were also found on *D. magna* and *C. dubia* with immobility as the endpoints (Isidori et al., 2005; Kim et al., 2007). SMX was once reported to have crossed the human placenta, and an increased number of micronuclei was observed in the bone marrow due to the combination of SMX with trimethoprim (IARC, 2001). All of these studies implicated the possibility of SMX to result in behavior and growth defects. In addition, behavior indicators showed better

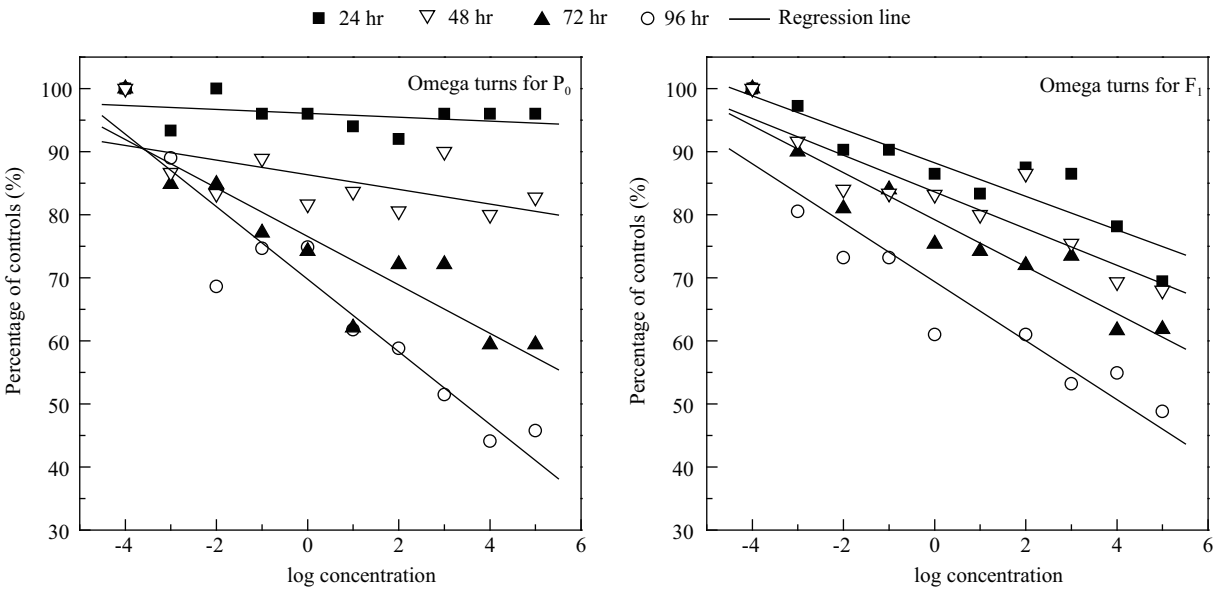

Fig. 4 Concentration-response relationship of Omega turns for P_0 and F_1. Concentration unit was μg/L before logarithm.

Table 3 Fitted linear functions and ECs of Omega turns for P_0 and F_1

	Exposed time (hr)	Functions	R^2	EC_{10} (μg/L)	EC_{15} (μg/L)	EC_{20} (μg/L)	EC_{25} (μg/L)	EC_{50} (μg/L)
P_0	24	$y = -0.31x + 96.09$	0.1352	>1.00E+05	>1.00E+05	>1.00E+05	>1.00E+05	>1.00E+05
	48	$y = -1.16x + 86.34$	0.3387**	1.11E-01	2.23E+00	4.48E+01	>1.00E+05	>1.00E+05
	72	$y = -3.84x + 76.55$	0.8171**	3.14E-04	6.30E-03	2.51E-01	3.95E-01	>1.00E+05
	96	$y = -5.75x + 69.79$	0.9073**	3.06E-04	2.26E-03	1.68E-02	1.24E-01	2.77E+03
F_1	24	$y = -2.66x + 88.24$	0.8398	2.18E-01	1.65E+01	1.25E+03	9.49E+04	>1.00E+05
	48	$y = -2.91x + 83.61$	0.8257	6.37E-03	3.33E-01	1.74E+01	9.09E+02	>1.00E+05
	72	$y = -3.73x + 79.23$	0.904	1.30E-03	2.84E-02	6.22E-01	1.36E+01	>1.00E+05
	96r	$y = -4.68x + 69.39$	0.8644**	<1.00E-04	4.62E-04	5.41E-03	6.33E-02	1.39E+04

* $P < 0.05$ vs. 24 hr-exposure, ** $P < 0.01$ vs. 24 hr-exposure.

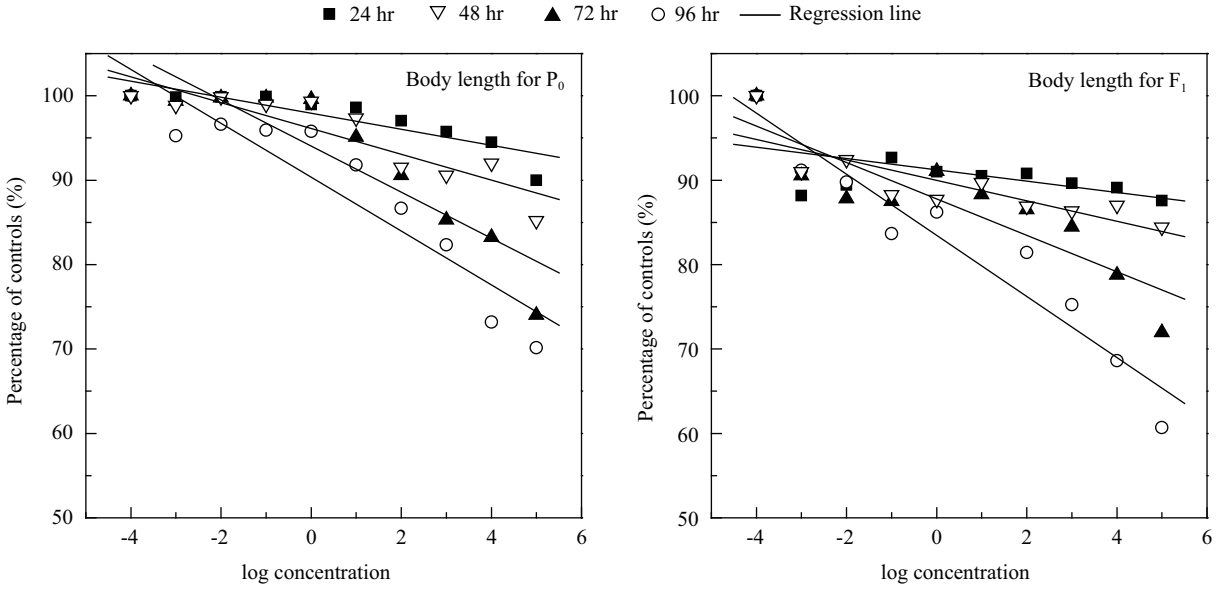

Fig. 5 Concentration-response relationship of body length for P_0 and F_1. Concentration unit was μg/L before logarithm.

sensitivity than growth indicator, which is consistent with previous studies (Wang et al., 2007; Wang and Wang, 2008).

The effects of SMX on the exposed parent generation were confirmed to be transferred to the unexposed progeny through the observation of the defects on the offspring as shown in Figs. 2, 3, 4 and 5. Although previous publications did not mention the potential of its transferable properties, some sidelight could be given by studies on the toxicities of SMX and its metabolites. Bhaiya et al. (2006) not only provided the evidence on the biological effects for the reactive metabolites of SMX, but also demonstrated that the reactive metabolites can provoke oxidative stress in a time- and concentration-dependent fashion in normal

Table 4 Fitted linear functions and ECs of body length for P_0 and F_1

	Exposed time (hr)	Functions	R^2	EC_{10} (μg/L)	EC_{15} (μg/L)	EC_{20} (μg/L)	EC_{25} (μg/L)	EC_{50} (μg/L)
P_0	24	$y = -0.95x + 97.92$	0.7852	>1.00E+05	>1.00E+05	>1.00E+05	>1.00E+05	>1.00E+05
	48	$y = -1.53x + 96.13$	0.8045	1.02E+04	>1.00E+05	>1.00E+05	>1.00E+05	>1.00E+05
	72	$y = -2.73x + 94.04$	0.824	3.02E+01	2.05E+03	>1.00E+05	>1.00E+05	>1.00E+05
	96	$y = -3.19x + 90.37$	0.8615*	1.31E+00	4.82E+01	1.78E+03	6.58E+04	>1.00E+05
F_1	24	$y = -0.67x + 91.22$	0.331	6.62E+01	>1.00E+05	>1.00E+05	>1.00E+05	>1.00E+05
	48	$y = -1.21x + 89.98$	0.6983	9.63E-01	1.31E+04	>1.00E+05	>1.00E+05	>1.00E+05
	72	$y = -2.16x + 87.78$	0.7727	9.38E-02	1.94E+01	4.00E+03	>1.00E+05	>1.00E+05
	96	$y = -3.62x + 83.47$	0.9226*	1.57E-02	3.78E-01	9.09E+00	2.19E+02	>1.00E+05

* $P < 0.05$ vs. 24 hr-exposure, ** $P < 0.01$ vs. 24 hr-exposure.

human dermal fibroblasts. Since *C. elegans* also contains multiple enzymes and the antioxidant system is also one typical defensive system for this nematode (Keaney et al., 2004), it was inevitable for the metabolites of SMX to arouse biological effects. In addition, the fact that SMX crossed the human placenta (IARC, 2001) made it more critical to consider the exposure time, which covered the whole processes when the second generation was formed (Hill et al., 2006), therefore the uterus was likely to serve as the pathway for SMX or its metabolites to get transferred into the progeny.

The concentrations employed in this study were environmentally relevant, and plenty of EC values for the exposed parent generation were at environmental concentration levels as shown in Tables 1, 2, 3, and 4. At the same time, the unexposed progeny also showed many EC values at environmental levels even though their parents were exposed to relatively low concentrations. Along with reported resistance to SMX (Chelossi et al., 2003; Le et al., 2005), current results suggest ecological risks of antibiotics be investigated systemically under environmental concentration levels.

4 Conclusions

C. elegans suffered behavior and growth effects in a time- and concentration-dependent fashion after exposed to SMX directly, and the effects were transferred to the unexposed progeny. Special attention should be paid on the defects and the transferred effects on *C. elegans* at environmentally relevant concentration levels. Further studies should focus on the mechanisms behind the transferable responses.

Acknowledgments

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