EFFECT OF SOME ENDOCRINE DISRUPTING CHEMICALS ON THE SEX-RATIO OF CAENORHABDITISELEGANS

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Abstract: Endocrine disrupting chemicals (EDCs) have been proved to concern on risk of ecosystem, wildlife and human health. Several biological assays and animal model have been developed to screen for the activities of the polluted EDCs. None of which has focused on the effects of EDCs on the sex-ratio of organism. This study proposed to use Caenorhabditis elegans as the model in laboratory system to screen for deleterious EDCs. Four types of EDCs were employed in the experiment including 4-Nonylphenol (NP), Tributyltin chloride (TBT), Triphenyltin chloride (TPT), and Testosterone (TS). Two sublethal concentrations were used; the LC_{20} (lethal concentration at 20% death) and half of the LC_{20} ($LC_{20}/2$). Two incubating temperatures regimes were set up; at 22°C (normal culture condition) and at 33°C for 4hrs (male-induced temperature) and then continue at 22°C. The fourth larval stage (L4) of a multi-generation (P0 – F9) was grouped and incubated under the combination of the sublethal concentration and the temperature regimes system. The worms were exposed to the targeted EDCs for 4 generations, P0 to F3, and without EDCs thereafter for 5 generations, F6 to F9. Control groups were incubated under the 2 regimes of temperature without EDCs exposure. Results showed the higher sex-ratio in the groups of worm exposed to EDCs in both of the temperature regimes. The system also exhibited the important biological activities of the targeted EDCs including the concentration-dependent, time-dependent, generation-dependent, temperature-dependent manner and recovery.

Keywords: Endocrine Disrupting Chemicals (EDCs), Nonylphenol (NP), Tributyltin chloride (TBT), Triphenyltin chloride (TPT), Testosterone (TS), sex-ratio, Caenorhabditis elegans

I. INTRODUCTION

Endocrine disrupting chemicals (EDCs) is widely recognized as a major environmental issue at present, with strong evidences for adverse effects in wildlife populations globally [1]. EDCs have been investigated in animals and humans with disturbing activities on the normal action of the endocrine system [2]. The effects range from disruption on human health [3, 4], developmental processes, to altered reproductive capacity, and abnormal behaviors [1]. In addition, exogenous sex hormones can change sexual determination, sexual differentiation, and expression of secondary sex characteristics in a variety of invertebrates and vertebrates [5]. Among such chemicals, 4 EDCs have been focused in this study including Nonylphenol (NP), Tributyltin Chloride (TBT), Triphenyltin chloride (TPT) and Testosterone (TS). NP is a degraded product of alkylphenol polyethoxylate and classified as nonionic surfactant employed in many detergent formulations for industrial and household use [6]. TBT and TPT are known to be included in the organotin compounds group. They have been widely used in agriculture, as fungicides, algaecide, molluscicide [7] and insecticides [8]. In addition, TS, the steroid hormone of male human and animal origin is frequently detected in environment [9]. Information on the impact of TS in the environment is limited, but the effect on aquatic organisms downstream from some industrial source have been reported including masculinization of female fish[10]. All of these EDCs have never been studied the effect on sex-ratio of *Caenorhabditis elegans*.

C. elegans has been used as a model organism in several biological sciences including soil ecology [11] and aquatic toxicology [12]. This nematode is a free-living organism, thin transparent body approximately 1 mm in length. Exists as either a self-fertilizing hermaphrodite or as a male, with simple developmental process highlight,*C. elegans* being easily monitored in laboratory. Comprehensive work that has been done on sequencing the genome of *C. elegans*attracted many scientists to study the disruption of several physiological functions [13]. Also, *C. elegans* is a majority to be use in studying on endocrine regulation and disruption as test organism [14], xenobiotic and ecotoxicological studies [15].

Male appearance in natural population of *C. elegans* is depended very much on some kind of environmental stresses, especially high temperature [16]. Increasing of individual male may influence on the outcrossing rate patterns of genetic variation, and the ability of natural selection to act within populations [15]. It is postulated that sex provides the advantage of generating novel, adaptive gene combinations and/or preventing the accumulation of deleterious mutation [17]. Investigation of sex ratio (male/hermaphrodite) in *C. elegans* populations might be used as one of toxicity test and have been developed to test the effects of xenobiotic chemicals [18]. *C. elegans* is a potent model for sex ratio evaluation because of the simple control condition could be set up in laboratory at 27 - 33°C [16, 19, 20, 21].

However, *C. elegans* sex-ratio was rarely used to interpret the effect of environmental EDCs [22].
Thus, the objective of this study was to examine the efficiency of sex-ratio in *C. elegans* to be used as indicator of EDC contamination at laboratory scale. Risky EDCs including NP, TBT, TPT and TS, with potentially polluted in terrestrial or aquatic ecosystem worldwide, were examined under a male-inductive temperature at 33°C.Influences of the target EDCs after withdrawal of the exposure was also investigated on recovery using sex-ratio under multi-generation cultures of the worm.

II. DETAILS EXPERIMENTAL

2.1. Chemicals and consumable items

The following compounds were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA): dimethyl sulfoxide (DMSO), NP, TBT, TPT, TS and all other culture-reagents. Culture plates and consumable items of all types were from Nunclon Nunc, Roskiide, Denmark.

2.2. Nematode stock culture and Synchronization methods

Stock culture of *Caenorhabditis elegans* (strain N2) were cultured at 22°C and maintained under standard conditions on Nematode Growth Medium (NGM) agar plates [23]. The bacteria stock (*Escherichia coli*, strain OP50) was used as food source for the worm. The worm and bacteria were obtained from the Human and Animal cells Technology research unit (HACT), Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.
C. elegans was prepared from a synchronized culture following the method of Hope[24]. Briefly, fully-grown culture of the worms were washed off plates using M9 Buffer (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 5 g/l NaCl, 0.25 g/l $MgSO_4.7H_2O$), placed into 15 ml conical tubes, and centrifuged at 800xg for 10 minutes at 4°C. The supernatant was drawn off the pellet and 10 ml of fresh M9 added. The nematode/egg pellet was suspended, centrifuged, and washed with M9 twice more for a total of three washes. The cleansed pellet was suspended in 10 ml of a basic hypochlorite solution (NaOCl) and agitated for approximately 5 min. The surviving eggs were centrifuged at 650xg after most adult carcasses were discarded, and the pellet washed 3 times with fresh M9 buffer. Washed eggs were removed from the conical tube and placed in batches on K- medium (3.1 g/l NaCl, 2.4 g/l KCl) tube, with unseeded of bacteria, to be hatched for 24 hr. Without food, *C. elegans* eggs hatched and arrested as L1 larvae [25]. The final pellet was re-suspended in 0.5 ml K-medium and transferred onto NGM plates inoculated with the bacteria.

2.3. Lethal concentration assay

The lethal concentration assay is a standard toxicological assay that allows to determine the concentration of chemicals at which 50% of the animals are killed (LC_{50}). The assay was performed according to procedure of Donkin and Williams [26]. In brief, 0.5%v/v DMSO in deionized water was prepared and used as a EDCs vehicle [27]. Stock solution of each EDCs was dissolved in the vehicle to get 10^{-2} M. Prepare 6-serial dilutions of each EDC liquid-mixture, ranging between 10^{-3} to 10^{-13} M, by dissolved each of the stock solution in K-medium. Pipette 1 ml of each EDCs liquid-mixture into each well of 24-well tissue culture plates. 15 young-adult (YA) worms were dispensed into each well at which performing triplicate of each concentration. Worms were exposed for 24hrs at 22°C without feeding. The number of dead and/or alive worms was determined by the absence of touch-provoked movement when probed with platinum wire under dissecting microscope[28]. The LC_{50} and the 20% lethal concentration (LC_{20}) were calculated using the PROBIT [29].

2.4. Sublethal toxicity assay for sex-ratio

In this study, sublethal concentration (sub-conc) of each EDC was defined into 2 categories the LC_{20} and half of the LC_{20} (i.e. $LC_{20}/2$). NGM in 3.5 mm plates were prepared by adding mixture of each EDCliquid-mixture to get the final concentrations at LC_{20} or $LC_{20}/2$. Once NGM setting, 300µl of *E. coli* OP50 concentration at $1.5x10^8$ viable cells per ml [30] (measured by OD at 600 nm [31]) was seeded on top and incubated at 37°C. After 24hrs, 300µl sub-conc of each EDC was added on top of *E. coli* and incubated for 6hrs. 5 worms at the 4th larval stage (L4) from synchronization method (see 2.2) were transferred to each plate using a worm-pick technique [32].
Two sets of NGM+EDCs plate with 5 L4-worms on each plate (as mentioned above) were prepared and incubated in the following 2 temperature regimes (1) the normal temperature of culture at 22°C (setting up as control groups), (2) the male-inductive temperature at 33°C for 4hrs and then 22°C afterward (abbv. 33/4-22°C). The total incubated duration of each temperature regime was 40hrs to get adult worms with laid eggs. After incubation, the 5 adult worms were discarded and the eggs were taken to continue on incubation for 30hrs to get L4-worms of the first filial generation (F1). Then 5 of the L4/F1 worms were randomly picked and transferred to each of the new NGM+EDCs plates, while the rests continued the incubation for another 18hrs before the sex-ratio recording on adult worms. The reason of using L4-worms to initialize the plate of each generation is their sensitivity of male-induction temperature [33].The incubation cycle of each temperature regime on EDCs exposed worms was repeated on F2 and F3 generations. From F4 to F9, the worms were investigated on the influent effects with recovery on

sex-ratio after the withdrawal of EDCs exposure. Four control groups were set up and investigated alongside with the EDCs exposed groups including the non-treated control and the vehicle control group, each of which taken under the two temperature regimes, 22°C or 33/4-22°C.

2.5 Statistical analysis

Results are presented as the mean ± standard error of the mean. Data was statistically evaluated by a Student's *t*-test. Determination of significant differences between means was performed at $p \leq 0.05$.

III. RESULS AND DISCUSSION

3.1. Lethal concentration assay

LC_{50} of each EDC on adult *C. elegans* is shown in Table 1. TBT and TPT exhibited the approximately similar toxicity at the level of 10^{-6} M, NP a lesser toxicity at 10^{-5} M and TS, the male sex-hormone, the least toxicity. NP was reported to enhance growth and reproduction of *C. elegans* at the concentration between 50-200 μg/l [6]. However, LC_{50} of NP on *C. elegans* in this experiment was differed from that reported previously at 5.0 $x10^{-3}$ M [34]. The LC_{50} of organotin compound (TBT and TPT) and TS was investigated using *C. elegans* previously, but the LC_{50} was not very clear presented [28, 34].Though, the present data in this study have confirmed on the result from those reports that *C. elegans* exhibited more sensitive to synthetic-chemicals (NP, TBT and TPT) than steroid hormone of TS.

Table 1. LCs (M) of each EDC on the adult *C. elegans*. Sublethal concentrations, at LC_{20}, with values rounded to whole numbers, were obtained for using in the evaluation of the EDCs effect on sex-ratio.

EDCs	LC_{50}	LC_{20}	$LC_{20}/2$
NP	$1.13x10^{-5}$	3.0×10^{-7}	1.5×10^{-7}
TBT	$1.48x10^{-6}$	9.0×10^{-8}	4.5×10^{-8}
TPT	$1.75x10^{-6}$	6.0×10^{-8}	3.0×10^{-8}
TS	$1.06x10^{2}$	1.0×10^{-7}	0.5×10^{-7}

3.2. Effect of EDCs on sex-ratio

To identify male from hermaphrodite *C. elegans*, the important morphological character of male, the copulatory bursa, was distinguished from the posterior tapering point tail and the vulva of female (Fig. 1). This bursa is also prominent in L4 male, but with smaller in size [35]. Picking up of L4 hermaphrodites (without existing of egg in uterus) to initialize the culture plates of each generation was based on these 2 sexual characteristics.

The effect of each EDC on the sex-ratio of *C. elegans* in multi-generation treatments is shown in Fig.2. NP showed significant increase of sex-ratio (i.e. increase number of male worms in the culture population) in the 22°C regime in the exposed generations (F1-F3), declined in F4 and recovery from F5 until the end of the experiment (Fig.2 A, B). In the 33/4-22°C regime with NP, sex-ratio was delayed during F1 and F3, but significantly peaked in F3 before rapidly declined in the withdrawal generations and stayed recovery just the same to the controls. It was obviously showed that the temperature shocked at 33°C for 4hrs in the control groups resulted in approximately 3-4 times higher male number in the population than those in the 22°C regime. Concentration-dependent manner of the NP effect was not very clear in both of the graph pattern of sex-ratio change between the LC_{20} (Fig.2 A) and $LC_{20}/2$ (Fig.2 B). This result support several of the previous literature experiments that accumulation of NP in sediment may cause toxicological effect to benthic fauna [36], on the sex-organ development in fishes [37] and even the risk of DNA damage effects to aquatic animals [38].

C. elegans showed high sensitivity to TBT at the LC_{20} (Fig.2 C). Astonishingly, in the 22°C regime, the peak of sex-ratio was last to F4 (withdrawal generation) before declining, while in the 33/4-22°C regime, highest sex-ratio initially found on F1 and gradually decreased until the end of the experiment. This is the supporting evidence of TBT temperature-dependent toxicity on aquatic organisms previously reported [39]. However, at lower concentration of TBT, $LC_{20}/2$ (Fig.2 D), the sex-ratio decreased by half, comparing to that of LC_{20} (Fig.2 C) at the 22°C regime, while in the 33/4-22°C regime, there was almost no significant difference of sex-ratio to those of the controls (Fig.2 D). This means that TBT activities displayed the concentration-dependent manner [40]. TBT activity was also reported with species sensitivity [41].

TPT, the same group of organotin compound to TBT, but expressed different effects on sex-ratio of *C. elegans* in this experiment. In the 22°C regime, peaks of TPT activity was at the last exposed generation, F3 (Fig.2 E, F), and declined thereafter until with full recovery from F6 to F9 without a clear concentration-dependent manner. Temperature-dependence was also observed on TPT in similar manner to those of TBT. In the 33/4-22°C regime, the non-significant difference of the LC_{20} exposed worms to the controls (Fig. 2 E), expressed the complete inhibition of male-induction in *C. elegans*. This is the observation for the first time of toxicity adverse effects of TPT on organism, using experimental model in laboratory. Sex-ratio change due to the exposure to TPT was reported under normal condition of natural environment of several species of wild life, for examples – copepod [42], marine medaka [43] and whelk [44].

The effect of TS on C. elegans sex-ratio was similar to those of NP, but with slower recovery (Fig.2 G, H). Recovery of *C. elegans* in the 33/4-22°C regime was not complete with higher sex-ratio above the controls, in comparison to those at the 22°C regime. This may be because of the strengthening of TS activity to the male-induction using temperature shock in the 33/4-22°C regime. Exposed to TS at LC_{20} (Fig.2 G) or

$LC_{20}/2$ (Fig.2 H) exhibited almost the similar responses, explaining possibly that this hormone works at an activity threshold without concentration-dependent in *C. elegans*[45].

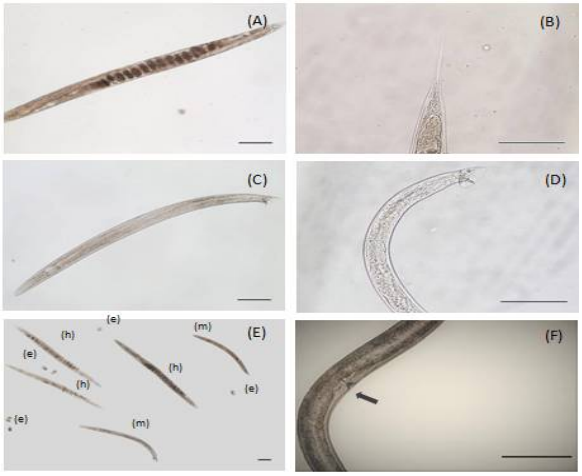


Fig. 1 Morphogenesis images of *C. elegans* showing adult hermaphrodite (A); posterior of hermaphrodite (B); male (C); posterior part of male with copulatory bursa (D), low magnified view of the 4 stages (E), hermaphrodite (h), male (m), and eggs (e); and vulva of L4 (F). Scale bar = 100µm.

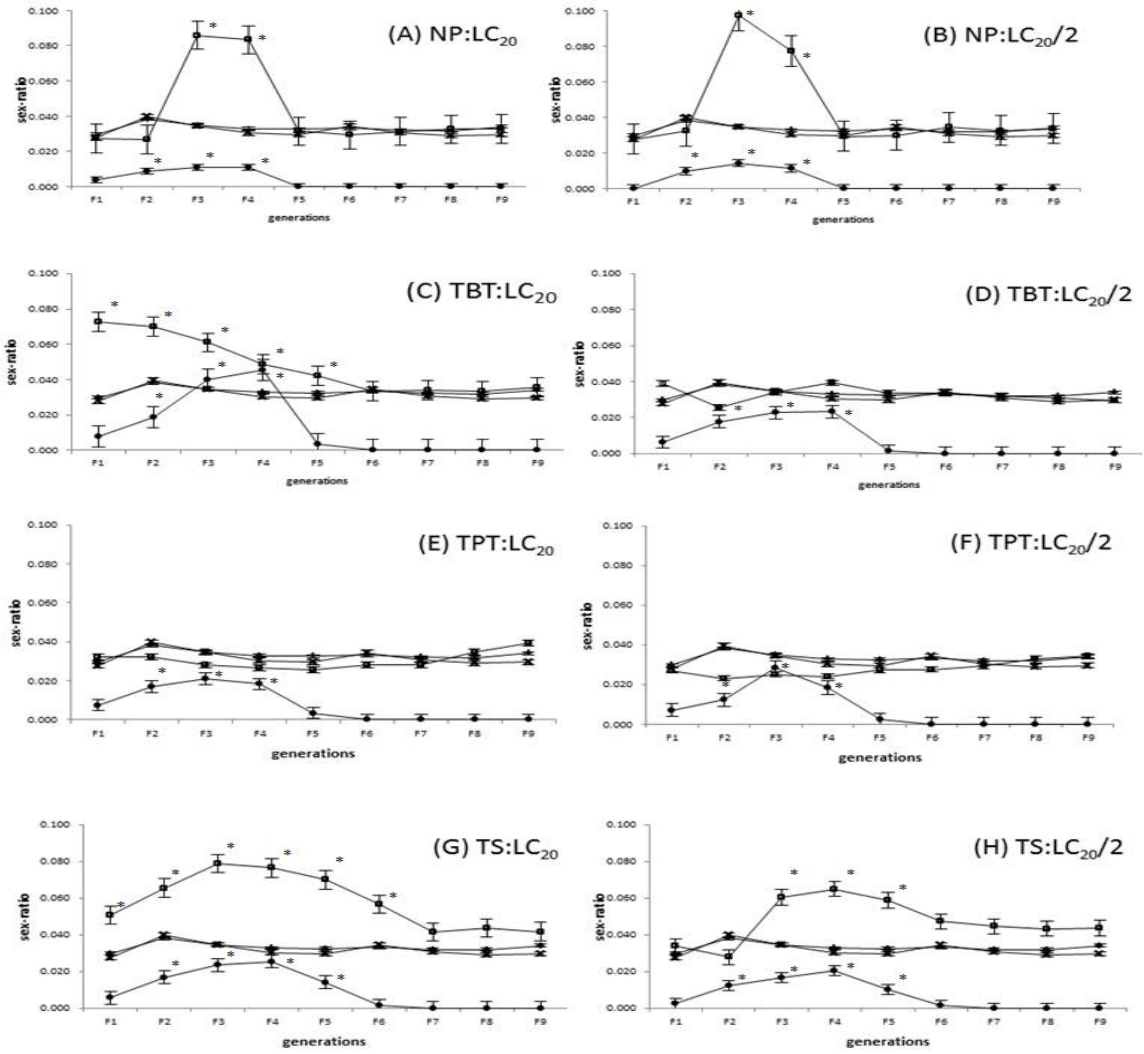


Fig.2. Effect of EDCs on the sex-ratio in multi-generations of *C. elegans*. Exposed generations to EDC was F1 – F3, while F4 – F9 the withdrawal and recovery. In 22°C regime, only EDC 22°C (●) is presented, because the other 2 un-presented groups (the non-treated control 22°C and the vehicle control 22°C) showed none of male and so with zero sex-ratio. For the 33/4-22°C regime, all 3 groups are presented, non-treated control 33/4-22°C (+), vehicle control 33/4-22°C (x) and EDC 33/4-22°C (□). Please notice that the same 2 lines of control groups in the 33/4-22°C regime are presented in all graphs for a convenience comparison. Significant different of the mean (*) in the 22°C regime was recognized in comparison to that of in F1 of EDC 22°C, while in 33/4-22°C regime compared to that of the non-treated control 33/4-22°C of the same generation.

CONCLUSION

In the system of *C. elegans* in this experiment, sex-ratio was proved to be used as effective indicator of EDCs contamination at the laboratory scale. Nonylphenol (NP), Tributyltin Chloride (TBT), Triphenyltin chloride (TPT) and Testosterone (TS) were examined under the 4 controllable conditions; the chemical concentrations, the developmental stages and filial generations of the worms, the normal culture temperature at 22^{o}C regime, and the higher stress temperature of male-induction at 33/4-22^{o}C regime. The model system showed the enhanced or inhibited properties of the targeted EDCs on the worm sex-ratio. The biological activities of the targeted EDCs could be evaluated under the system including the concentration-dependent, time-dependent, generation-dependent, temperature-dependent manner and recovery. The accuracies, simple to handles, and with low cost equipment pushing this model as one of the attractive system to be employed in any preliminary study of EDCs.

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