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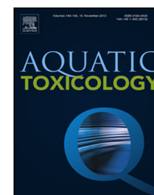

# Proteomic changes in brain tissues of marine medaka (*Oryzias melastigma*) after chronic exposure to two antifouling compounds: Butenolide and 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT)

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ABSTRACT

SeaNine 211 with active ingredient of 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT) has been used as a “green” antifouling agent worldwide but has raised serious biosafety concerns in coastal environments. DCOIT has the potential to disrupt the neurotransmission in nervous system, but the underlying mechanism has not been clarified. In the present study, we used TMT six-plex labeling coupled with two-dimensional LC-MS/MS analysis to investigate the protein expression profiles in brain tissues of the marine medaka (*Oryzias melastigma*) after a 28-day exposure to environmentally-realistic concentration of DCOIT at 2.55 μg/L (0.009 μM) or butenolide, one promising antifouling compound, at 2.31 μg/L (0.012 μM). DCOIT and butenolide induced differential expression of 26 and 18 proteins in male brains and of 27 and 23 proteins in female brains, respectively. Distinct mechanisms of toxicity were initiated by DCOIT and butenolide in males, whereas the protein expression profiles were largely similar in females treated by these two compounds. In males, DCOIT exposure mainly led to disruption of mitogen-activated protein kinase (MAPK) signaling pathway, while butenolide affected proteins related to the cytoskeletal disorganization that is considered as a general response to toxicant stress. Furthermore, a sex-dependent protein expression profile was also noted between male and female fish, as evident by the inverse changes in the expressions of common proteins (5 proteins for butenolide- and 2 proteins for DCOIT-exposed fish). Overall, this study provided insight into the molecular mechanisms underlying the toxicity of DCOIT and butenolide. The extremely low concentrations used in this study highlighted the ecological relevance, arguing for thorough assessments of their ecological risks before the commercialization of any new antifouling compound.

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## 1. Introduction

In the marine environment, coating immersed surfaces with antifouling paints is a common approach to deter the undesirable attachment of marine organisms (Callow and Callow, 2002; Qian et al., 2013). Since the ban of organotin-based antifouling coatings for the sake of ecological safety, various organic booster biocides, such as Irgarol 1051 and SeaNine 211, have been used in combination with cuprous oxide (Konstantinou and Albanis, 2004; Voulvoulis, 2006).

SeaNine 211, containing active biocide 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT) (Fig. 1; Molar mass: 282 g/mol), is marketed as an environmentally acceptable alternative to organotin due to its rapid degradation and efficacious antifouling activity (Shade et al., 1993; Jacobson and Willingham, 2000). However, recent studies show the non-negligible threats of SeaNine 211 such as its accumulation in coastal water and high toxicity to marine organisms (Omae, 2003; Konstantinou and Albanis, 2004; Thomas and Brooks, 2010). Pollution of SeaNine 211 has been reported in various environmental samples, such as seawater and sediment along the coast (Konstantinou and Albanis, 2004). For

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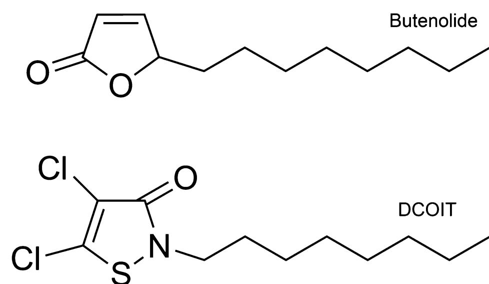


**Fig. 1.** The chemical structures of butenolide and DCOIT.

example, above 3.3 μg/L SeaNine 211 has been detected in the seawater around Spain marinas (Martínez et al., 2000). Such high level of accumulation could pose serious environmental effects. It is reported that exposure to SeaNine 211 at as low as 0.1 ng/L caused significantly delayed growth of sea urchin eggs (Kobayashi and Okamura, 2002). Low level of SeaNine 211 also inhibits ATP synthesis in the mitochondria prepared from rat liver (Bragadin et al., 2005), and suppresses the immune function in tunicate haemocytes (Cima et al., 2008). Furthermore, increased apoptosis in the testicular germ cells of mummichog *Fundulus heteroclitus* is induced by chronic exposure to 1 μg/L SeaNine 211 (Ito et al., 2013). A 28-day exposure of marine medaka (*Oryzias melastigma*) to 2.55 μg/L DCOIT results in impaired reproductive success, as evident by the greatly imbalanced sex hormones ratio ($E_2$/T) in male fish, but no effect in female fish (Chen et al., 2014). Neurotoxicity is a particular concern associated with DCOIT exposure which decreases acetylcholinesterase (AChE) activity in the brains of marine medaka (*Oryzias melastigma*), suggesting the potential disturbance on neurotransmission (Chen et al., 2014).

In contrast, butenolide [5-octylfuran-2(5H)-one] (Fig. 1; Molar mass: 196 g/mol) has been recently patented and being proposed as a promising antifouling agent and is the least toxic among the antifouling biocides against non-target organisms at various trophic levels (Zhang et al., 2011; Chen et al., 2014). To elucidate and compare the possible neurotoxicity of these antifouling compounds and the underlying molecular mechanism, we examined and compared the brain proteome perturbation of marine medaka after 28 days' chronic exposure to extremely low concentrations of DCOIT (2.55 μg/L, 0.009 μM) and butenolide (2.31 μg/L, 0.012 μM). We also revealed the gender differences in response to these two antifouling compounds.

## 2. Materials and methods

### 2.1. Chemicals

DCOIT (purity > 99%) was purchased from Waterstone Technology (Carmel, IN, USA). Butenolide (purity > 99%) was obtained from Medicilon (Shanghai, China). Stock solutions of DCOIT and butenolide were prepared in high performance liquid chromatography (HPLC)-grade dimethyl sulfoxide (DMSO) (Sigma–Aldrich, St. Louis, MO, USA). Primary antibody against 14-3-3 gamma protein was purchased from Abcam (Cambridge, MA, USA). TMT mass tagging kit was obtained from Thermo Scientific (Rockford, IL, USA).

### 2.2. Fish maintenance and exposure

The maintenance and exposure of adult marine medaka (*O. melastigma*) were conducted as described by Chen et al. (2014) in accordance with the Code of Practice for Care and Use of Animals for Experimental Purposes developed by the Animal Welfare Advisory Group and produced by the Agriculture, Fisheries and Conservation Department (Hong Kong). Briefly, adult medaka (4-month old) were cultured in fully aerated and charcoal-filtered artificial seawater (salinity: 30‰) with constant temperature at 24 ± 0.5 °C and 14 h: 10 h light: dark cycle. The fish were fed once daily with newly hatched *Artemia* nauplii and twice daily with flake food (AX5; Aquatic Ecosystems, USA). After 2-week acclimation in 13 L tanks containing 10 L seawater, the fish (12 males and 12 females per tank) were exposed to nominal 3.0 μg/L DCOIT (0.011 μM) and 3.0 μg/L butenolide (0.015 μM) with DMSO concentration at 0.001%, while the control group only received 0.001% DMSO. The measured concentrations of DCOIT and butenolide in the seawater were 2.55 μg/L (0.009 μM) and 2.31 μg/L (0.012 μM), respectively (Chen et al., 2014). The concentration of DCOIT used in this study is environmentally realistic because > 3.3 μg/L SeaNine 211 has been detected in the seawater in Spain marinas (Martínez et al., 2000). Each group included three replicate tanks and the exposure continued for 28 days. The seawater in each tank was half-renewed daily in the semi-static system to maintain constant exposure concentrations. After the exposure, the fish were randomly selected from the tanks and anesthetized in 0.03% MS-222. The brains tissues were collected and immediately frozen in liquid nitrogen prior to storage at −80 °C for further analysis.

### 2.3. Proteomic analysis

#### 2.3.1. Protein extraction and labeling

For proteomic analysis, brain tissues from 4 fish were pooled and homogenized on ice in lysis buffer (8 M urea and 40 mM HEPES, pH 7.4) using a Misonix Sonicator-XL2020 (Misonix, NY, USA). After centrifugation at 15,000 × g for 15 min at 4 °C, the supernatant was transferred into a new tube and the protein was precipitated using methanol and chloroform (Wessel and Flügge, 1984). The protein pellet was reconstituted in lysis buffer and quantified using RC-DC protein assay (Bio-Rad, Hercules, CA, USA). Afterwards, 150 μg of protein were taken from each sample and reduced using 5 μL of 200 mM triscarboxyethyl phosphine hydrochloride (TCEP) at 55 °C for 1 h. The reduced protein was then alkylated by incubation with 5 μL of 375 mM iodoacetamide for 30 min at room temperature in the dark. The protein solution was then diluted by 8-fold and subjected to tryptic digestion using 5 μg trypsin (Promega, Madison, WI, USA) per sample for 16 h at 37 °C. The peptide solution was acidified using trifluoroacetic acid (TFA), desalted using Sep-Pak C18 cartridges (Waters, Milford, MA, USA) and dried in a SpeedVac (Thermo Electron, Waltham, MA, USA). They were then resuspended in 100 mM triethylammonium bicarbonate (TEAB). The labeling of peptides using TMT 6-plex label reagents was performed at room temperature for 1.5 h (male brain: TMT agent 126 for control group, 127 for butenolide group, and 128 for DCOIT group; female brain: 129 for control group, 130 for butenolide group, and 131 for DCOIT group) according to the product manual. After the reaction was quenched by 5% hydroxylamine, six samples were pooled and dried in a SpeedVac.

#### 2.3.2. Strong cation exchange (SCX) fractionation of peptides

The dried peptide pellets were resuspended in 110 μL of buffer A (10 mM $KH_2PO_4$ and 25% acetonitrile, pH 3.0) and fractionated in the polySULFOETHYL A column (200 × 4.6 mm, 5 μm, 200 Å) (PolyLC, MD, USA) on Waters Delta 600 HPLC unit (Waters, Milford, MA, USA) (Han et al., 2013). An increasing gradient of buffer B (10 mM $KH_2PO_4$, 500 mM KCl and 25% acetonitrile, pH 3.0) was used to fractionate the peptide during the 60-min gradient elution protocol (100% buffer A for 10 min, 0%–30% buffer B for 25 min, 30%–100% buffer B for 5 min, and 100% buffer B for 10 min) at flow rate of 1 mL/min. The chromatography was monitored at 280 nm using the diode array detector and the peptide fractions were collected automatically. The chromatography at 280 nm showed that the majority of peptides were eluted from 22.5 min to 47.5 min

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