Effects of Reconstituted Deep Seawater on Detoxification of Organotin-intoxicated *Euglena gracilis* Z

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Abstract

Since deep seawater (DSW) is rich in minerals, contains few microbes, and exhibits physiological functionality, it is being considered as a potential water resource for the 21st century and presently used in several areas including functional food, aquaculture, food industries and medicine. Using unicellular organism, *Euglena gracilis*, as a biomaker, we examined the detoxification effects of reconstituted DSW that was brought about by desalination and subsequent reassemble of major constituents of mineral in raw DSW (referred as RDSW) on biota intoxicated with hazardous chemicals. After removal of NaCl, DSW was sterilized by filtration. *E. gracilis* Z strain (plant model) was grown heterotrophically under a light/dark regime at 28℃. The *Euglena* cell motility was impaired, and morphology altered by exposure to the toxicant tributyltin chloride. The cells were then incubated in desalinated deep seawater for 180 min, and restoration of cell motility and morphology were estimated by videomicroscopy. Distinct restoration effect in *E. gracilis* Z was observed after incubation with RDSW. The effects were largely attributed to trace minerals that are involved in the biotransformation system and cell motility.

Key words – mineral, reconstituted deep seawater, detoxification, organotin, *Euglena gracilis* Z

Abbreviations: TBTCl, tributyltin chloride; TBTO, tributyltin oxide; ICP, inductively-coupled plasma atomic emission spectrometry; RDSW, reconstituted deep seawater.

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Introduction

Deep seawater is being considered as a potential water resource to be used in several areas including functional food, aquaculture, food industries and medicine. Deep seawater is derivative from 200 m to 300 m below the mixing layer and the photic zone of the ocean. Without sunlight there is no primary productivity, therefore fewer living organisms are found to be existing, resulting in a virtually pathogen-free environment. The deep seawater also has significantly elevated levels of minerals, inorganic nitrates, silicates and phosphates compared to the nutrient deficient surface water. These nutrients are necessary for sustained growth of micro and macro organisms.

A number of minerals play an important role in certain cellular functions such as xenobiotic metabolism, intracellular signal transduction, energy metabolism and cell movement[1]. Using the phytoplankton *Euglena gracilis* as a model organism, we have been studying the effects of several mineral solutions that show some interesting physiologically active function particularly on the detoxification of tributyltin chloride (TBTCl)-intoxicated cells[2), 3), 4), 5)]. Organotin compounds, such as TBTCl have been used as antifouling agents on fishing net and boat hulls[6), 7), 8)], but their wide-spread use has contributed to contamination of the marine environment. Although the use of organotin compounds has been banned in Japan, they are still widely used in under-developed countries as has been evident from the detection of their residues in the marine environment[9), 10)]. Furthermore, it has been recently discovered that organotin is an endocrine disrupter of ecosystems at an extremely low concentration[10), 11), 12)]. However, an effective remedy that may decrease the toxic level of toxicant has so far not been found.

Euglena gracilis Z and achlorophyllous SMZ strains have been used as the model organism for TBTCl intoxication as biota is thought to be affected by TBTCl pollution in the ecosystem and as this unicellular organism is highly sensitive to the pollutant. It has been reported that *Euglena gracilis* is a sensitive test organism to test water quality[13), 14), 15)]. When *Euglena* cell is exposed to TBTCl, it loses cell maneuverability, then changes its shape from motile spindle form to spherical cyst form via teardrop form. Furthermore, as *Euglena* cell has the characteristics of both animal (SMZ strain) and plant (Z strain) cell, it can be used as a biomaker for both models in bioassay.

Earlier, we reported that the intoxication of *Euglena* cells by TBTCl and its detoxification by high electric field loaded water[2), 16)], and several solutions including mineral-encaging zeolites promoted the excretion of tin compounds from TBTCl-intoxicated *Euglena* cells, where the enhancing of the regeneration of the flagellum and accelerating the restoration of cell motility were observed[17)]. Previous results have suggested that minerals play critical roles in the restoration of the intoxicated cells[7), 16)].

In the present study, we compared invigoration effect of reconstituted deep seawater after desalination, or reconstituted DSW (RDSW) and speculated their restoration effect.

Materials and Methods

Reconstituted DSW and chemicals

RDSW with 50, 250 and 1000 of hardness were kindly provided by Akō Kasei Co., Ltd., Ako, Hyogo Pref. Japan. RDSW, were filtered through 0.45 μm filter before use. The detailed preparation procedure of RDSW is not opened.

TBTCl (ca. 95% purity) used as a model pollutant was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). TBTCl used in all experiments was prepared by the procedure as described in the previous paper[16]. To examine the effect of a chelator on the restoration of cell motility, a sufficient amount of Chelex-100® resin (BioRad Laboratories, Hercules, CA, U.S.A.) to trap the mineral was added to each RDSW solutions. After treating water species with the chelator, the water was filtered through No.2 filter paper to remove the Chelex-100® resin.

Model organism

A wild strain of *Euglena gracilis* Z was cultured in a Koren-Hutner medium[18] at 28℃ under illumination (2,800 lx) with a 12 hour light-on-off interval for 7 days. The cells used for these experiments were collected at the early stationary phase.

Evaluation of restoration of cell morphology and motility

The restoration of cell morphology and motility was examined by a video-microscope equipped with an image-analyzing system. Morphology and motility of cells after 0, 30, 60, 90, 120, 150 and 180 minutes of incubation were examined microscopically by an inverted microscope (Olympus type IMT-2) equipped with a video image analyzer (ARGUS-100, Hamamatsu Photonics Co., Hamamatsu, Shizuoka, Japan). Optimas data processing software (Optimas Corp., Bethell, Washington, 98011 U.S.A.) was used to count motile cells (spindle form) on at least 10 different video images, before the restoration efficiency was calculated and expressed in percent as the motile cell number / total cell number over time. Detailed experimental procedure was described in the previous paper[18].

A statistical analysis was carried out by using a Q-test and Student's t-test. P-values of <0.01 and <0.05 were considered significant.

Analysis of elements in RDSW

The composition of elements in RDSW was analyzed by ICP and ICP-MS with SPQ9000 plasma quadrupole mass analyzer apparatus (Seiko Instruments INC., Japan) according to the analytical method described by Sugiyama *et al*[19].

Results

Effect of RDSW for recovery of cell motility in TBT-intoxicated *Euglena* cell

Evaluation of restoration of cell morphology and motility

Figure 1 shows the recovery of cell motility in TBTCl-intoxicated *E. gracilis* Z strain due to RDSW. After 180 min, a remarkable recovery were observed in *E. gracilis* Z strain that had been incubated in hardness 1000 (59.4 %), 250 (52.3 %) and 50 (47.8 %) of RDSW. Recovery of the control was 14.4 %.

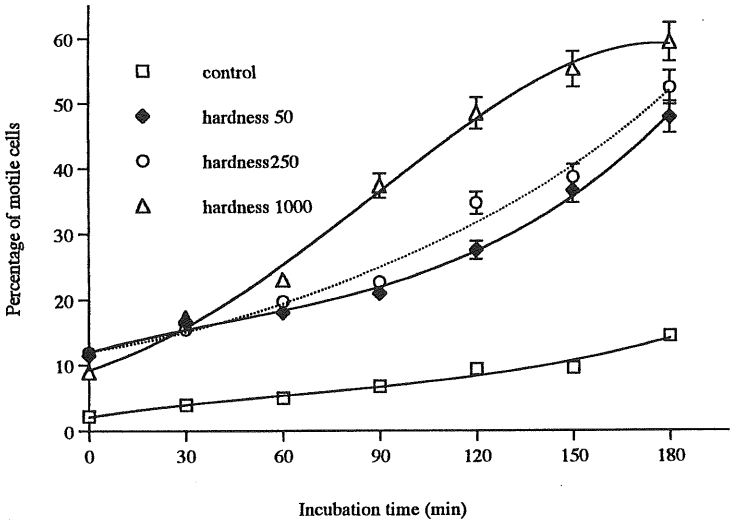

Fig. 1. Effect of RDSW on the restoration of cell motility of TBT-intoxicated *Euglena gracilis* Z

Each plot represents the restoration of the TBTCl-intoxicated immotile cyst form to motile spindle form a presented as percentage of the total cell number. Percentage motility was calculated by [motile cells/total cell count]× 100. □ motile cell percentage incubated in doubly distilled water as the control; ◆ 50 hardness of RDSW; ○ 250 hardness of RDSW; △ 1000 hardness of RDSW. Throughout the present study counting of motile and immotile cell numbers was made with at least 10 different video images in which 50 to 100 cells were included. Data in the figures were expressed as mean ± SD for 10 measurements.

Effect of chelator on the restoration of cell motility by RDSW

To examine whether the detoxification effect of RDSW on *E. gracilis* Z was due to minerals, RDSW was preliminarily treated with a potent chelator, Chelex-100®, which acts as a chelating groups in binding polyvalent metal ions. As shown in Fig. 2, RDSW lost its recovery effect on Z strain of *Euglena* cell when it was treated with the chelator prior to incubation.

Since RDSW appeared to play an important role in the restoration of cell motility with an effect that largely depended upon the constituent mineral, major mineral composition of the RDSW was analyzed by ICP-MS. The major elements detected in RDSW (hardness of 50) were Mg (200 ppm), Ca (71 ppm), K (69 ppm) and Na (74 ppm), and they were trapped by treatment with Chelex-100®. Iron in the RDSW was 0.4 ppb.

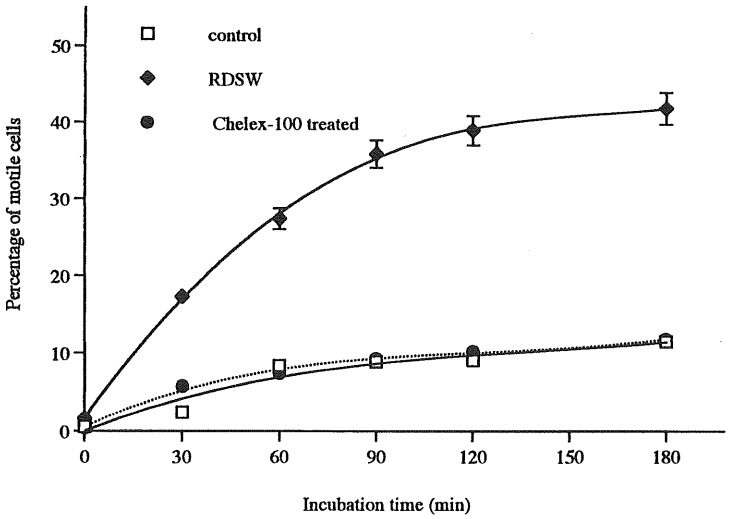

Fig. 2. Recovery of cell motility by TBTCl-intoxicated immotile *Euglena gracilis* Z in RDSW and chelated RDSW.

□ control; ◆ RDSW; ○ RDSW chelated by Chelex-100.® Doubly distilled water was used for the control.

Participation of minerals in the restoration of cell motility

Participation of Mg and Ca in the restoration of cell motility is obvious, however, it is still uncertain whether the effect was simply dependent on those minerals by themselves or not. Addition of different concentrations of Mg (as $MgCl_2$) and Ca (as $CaCl_2$) ranging from 0.1 ppm to 100 ppm as metal ion concentration to the incubation system in place of RDSW was made to examine their restoration effect. The sole addition of $CaCl_2$ was not as effective as the RDSW in the restoration of cell motility (Fig. 3). In contrast to the remarkable restoration of cell motility by the RDSW, the addition of Mg in the form of $MgCl_2$ at 100 ppm had a cell recovery of only 18 %. Similar result was obtained with $CaCl_2$ (data not shown), where sole addition of $CaCl_2$ was not as effective as with RDSW.

Furthermore, to make clear whether the restoration effect was dependent on Ca and Mg mixture, the recovery of cell motility was investigated with mixture of Mg and Ca. As shown in Fig. 4, a high recovery of the cell motility of TBTCl-intoxicated *E. gracilis* was observed at 180 min by the mixture of Ca and Mg. The incubation with the mixture of Ca and Mg was effective as same as the RDSW indicating the recovery of the TBTCl-intoxicated *E. gracilis* to bet due to the conbinatorial effect of the minerals.

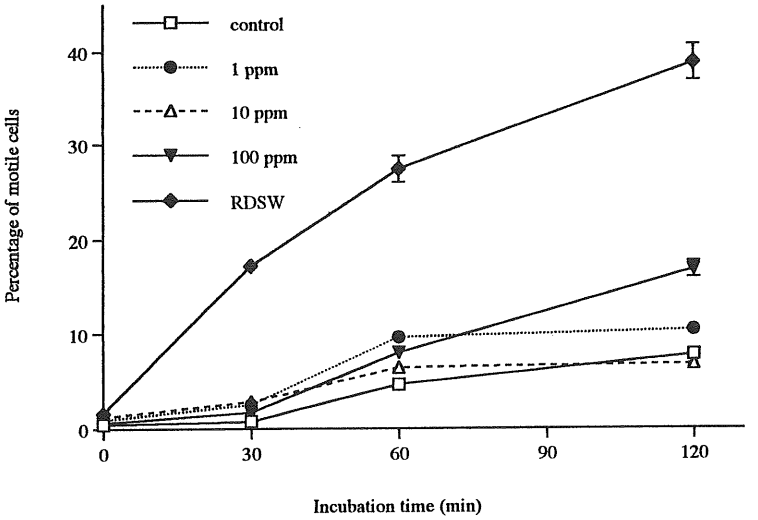

Fig. 3. Recovery of cell motility of TBTCl-intoxicated immotile *Euglena gracilis* Z by $MgCl_2$
Participation of Mg in recovery of cell motility was examined by comparing with RDSW and $MgCl_2$ of different concentrations with 1, 10 and 100 ppm as Mg. Doubly distilled water was used as the control.

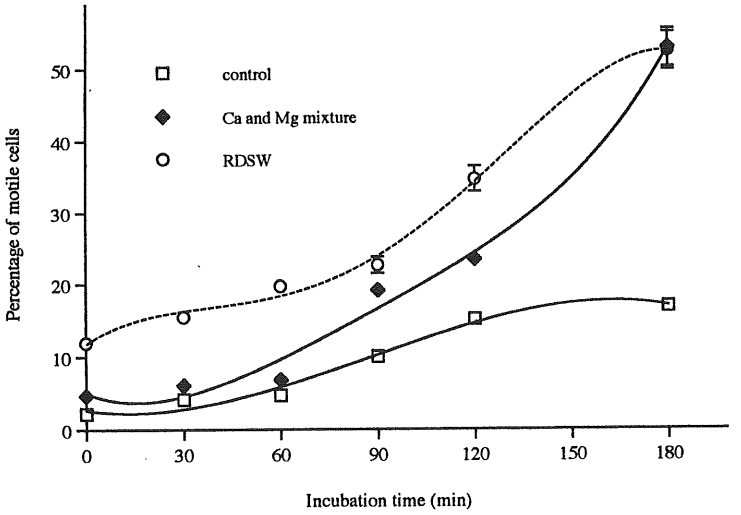

Fig. 4. Recovery of cell motility of TBTCl-intoxicated immotile *Euglena gracilis* Z by mixture of Ca and Mg
The concentration of the Ca and Mg in the mixture was 78 ppm of $CaCl_2$ and 10 ppm of $MgCl_2$ as Ca and Mg. Doubly distilled water was used as the control.

Discussions

In order to regenerate the flagellum and to reform the cell morphology to the motile spindle form, detoxification of TBTCl and excretion of tin compounds from the cell is not only firstly required but also of high importance. To regenerate the flagella in resting cells, it has been reported that the participation of minerals, especially iron, Ca^{2+} and Mg^{2+}, is necessary[20),21)]. The major elements in RDSW analyzed by ICP-MS were Ca and Mg and treatment of RDSW with a chelator reduced its recovery effects (Fig. 2). The result suggest that Ca^{2+} and Mg^{2+} play important roles during the TBTCl biotransformation and invigoration of cell function in *Euglena gracilis*. Similar result was obtained in our previous work with alternative current high voltage loaded water in the presence of wood ceramic[2)], in which Ca^{2+} and Mg^{2+} play a key role. However, the simple addition of Ca^{2+} or Mg^{2+} to the distilled water did not show a significant recovery effect on the TBTCl-intoxicated *Euglena* cell (Fig. 3). On the other hand, the recovery of the cell motility of TBTCl-intoxicated *E. gracilis* Z cell by RDSW and incubation with the mixture of Ca^{2+} and Mg^{2+} was the same at 180 min. The rapid recovery of cell motility by RDSW in *E. gracilis* means rapid exclusion or detoxification of TBTCl out of the cell. The remarkable restoration of motility by RDSW to TBTCl-intoxicated *Euglena* cells suggests that Ca^{2+} and Mg^{2+} or other trace minerals may contribute to the biotransformation and excretion of TBTCl or regeneration of flagella, directly or indirectly. On the other hand, iron as trace mineral should also play crucial role in the first stage of cytochrome P450 mediated biotransformation. In the present study we did not examine the effect of iron as divalent or trivalent form. To specify the roles of minerals especially of iron as a trace element contained in RDSW, further study is essential. As has been reported by Kinuta *et al.*[22)], calcium supplementation increases aromatase activity. Furthermore, Marc *et al.*[23)] reported that Ca^{2+} mediated the action of intoxication by phenobarbital probably via Ca^{2+}/calmodulin-dependent protein kinase, also dramatically inhibited the induction of the cytochrome P-450 genes.

The data obtained in the present study address the importance of RDSW exhibiting the recovery effect in restoring the motility of TBTCl-intoxicated *Euglena* cells. Although detailed mechanism of the recovery effect of RDSW is not yet finally elucidated, RDSW can be a remedy for detoxification of TBTCl intoxicated biota ranging from plankton to mammals.

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