2、4ジブロモフェノールと2、4、6トリブロモフェノールによるエゾバフンウニ幼生の生残と変態の阻害

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Inhibition of Larval Survival and Metamorphosis of the Sea Urchin *Strongylocentrotus intermedius* by 2,4-dibromophenol and 2,4,6-tribromophenol

Jing-Yu Li¹,², Hikaru ENDO¹, Yukio AGATSUMA¹, * and Kazuya TANIGUCHI¹

**Abstract:** We examined the inhibitory effects of 2,4-dibromophenol (DBP) and 2,4,6-tribromophenol (TBP), which are released by the kelps *Eisenia bicyclis* and *Ecklonia kurome*, on survival and metamorphosis of larvae of the sea urchin *Strongylocentrotus intermedius*. Almost all larvae were fallen in the presence of 5 and 10 ppm, and died in the presence of 20 and 50 ppm DBP and TBP after 24 h, indicating that DBP and TBP have strong toxicity for the larvae of *S. intermedius*. The percentage of larvae that underwent metamorphosis in filtered seawater after 1 h of exposure to a one-half dilution of saturated dibromomethane solution (~60 ppm) as a chemical inducer reached 43% after 1 h and rose to 75% after 24 h. However, the percentage of metamorphosed larvae in filtered seawater containing 10 ppm DBP was reduced markedly to 25-34% within 24 h, and that in filtered seawater containing 10 ppm and 20 ppm TBP was reduced markedly to 24-34% and 4-10%, respectively, within 24 h. All larvae exposed to 20 ppm and 50 ppm DBP and to 50 ppm TBP died after 1 h. These findings suggest that DBP and TBP have a strong inhibitory effect on larval survival and metamorphosis for *S. intermedius*. These bromophenols may play an important role in the chemical defense of kelps against sea urchin recruitment in the field.

**Key words:** Inhibitory effect; Metamorphosis; Survival; Bromophenol

In rocky subtidal areas, high densities of metamorphosed and settled juveniles of the sea urchins *Strongylocentrotus purpuratus* (Cameron and Schroeter 1980; Rowley 1989), *S. franciscanus* (Cameron and Schroeter 1980; Rowley 1989), *S. nudus* (Sano et al. 1998), and *S. droebachiensis* (Balch and Scheibling 2000) are found in crustose coralline communities. By contrast, few metamorphosed juveniles of *S. nudus* and *S. droebachiensis* are found in forests of *Eisenia bicyclis* (Sano et al. 1998) and *Laminaria longicruris* (Balch and Scheibling 2000), respectively. Post-settlement mortalities of the sea urchins *Evechinus chloroticus* and *S. purpuratus* are high in forests of *Ecklonia radiata* and *Macrocystis pyrifera*, respectively (Andrew and Chat 1985; Rowley 1990). The low rate of recruitment in kelp forests has led to the suggestion that kelp forests act as larval filters by harbouring species that consume larvae as they drift through the forest or settle on the bottom (Pearse et al. 1970; Bernstein and Jung 1979; Tegner and Dayton 1981; Dayton and Tegner 1984; Gaines and Roughgarden 1987; Harrold and Pearse 1987; Chapman and Johnson 1990).

Taniguchi et al. (1994) and Agatsuma et al. (2006) have reported that dibromomethane, a volatile metabolite produced by coralline algae (Itoh and Shinya 1994), strongly and rapidly induces metamorphosis of *S. nudus* and *S. intermedius*, suggesting that crustose coralline areas are nursery grounds for sea urchins. By contrast, our latest findings (Agatsuma et al. 2008)
have shown that 2,4-dibromophenol (DBP) and 2,4,6-tribromophenol (TBP), the volatile metabolites released from the kelps *Eisenia bicyclis* (Shibata et al. 2006), strongly inhibit the metamorphosis and survival of *S. nudus* larvae, resulting in failure of recruitment in kelp forests.

The sea urchin *S. intermedius* is found on intertidal and subtidal rocky bottoms in northern regions of Japan, down to Choshi, Chiba, on the Pacific coast, and to Toyama along the Sea of Japan coast (Shigei 1995). It is harvested commercially in Iwate, Aomori and Hokkaido, where the kelps *Saccharina* spp. and *Eisenia bicyclis* are dominant (Kawashima 1993). In Hirota Bay in southern Iwate Prefecture, *S. intermedius* inhabit in shallow waters where *E. bicyclis* grow (Li et al. 2008; Matsui et al. 2008). The population of zero-aged juveniles of *S. intermedius* is dense in small boulder areas with communities of small algae throughout the year in the field (Kawamura 1973). Settlement deterrents from kelps may play a major role in discouraging the settlement of sea urchin larvae in kelp forests (Agatsuma et al. 2008). This finding increases the possibility that DBP and TBP, which strongly inhibit the metamorphosis and survival of *S. nudus* larvae, are also active on *S. intermedius* larvae. The aim of the present study was to clarify the inhibitory effects of these bromophenols on survival and metamorphosis of *S. intermedius* larvae.

**Materials and Methods**

In this study, the inhibitory effects of the two bromophenols, 2,4-dibromophenol (DBP, Br₂C₆H₃OH) and 2,4,6-tribromophenol (TBP, Br₃C₆H₂OH) (Wako Pure Chemical Industries, Osaka, Japan) on survival and metamorphosis of *S. intermedius* larvae was examined. Experiments were conducted at Taneichi Office, Iwate Prefectural Fish Farming Center, in November 2005. Larvae of *S. intermedius* were reared at a density of approximately one individual per ml in a 0.5-m³ rectangular tank with a flow rate of 0.8 l/min and fed *Chaetoceros gracilis* at 50,000 cells/ml/day for about one week at a water temperature of 17-18°C. Light and dark conditions were controlled every 12 h. Larvae grown to the eight-armed stage with fully developed urchin rudiments were used in the present experiments.

**Survival of eight-armed larvae**

To test larval survival at different concentrations of DBP and TBP, 20 ml of seawater filtered to 5 μm containing approximately 20 eight-armed larvae was added to a Petri dish (5.5 cm diameter, 3.0 cm deep). Simultaneously, 0.4 ml of 3500, 1400, 700, 350, or 70 ppm DBP or TBP dissolved in 99.5 vol% ethanol (Wako Pure Chemical Industries) and 7.6 ml of 5-μm-filtered seawater were added. Thus, 28 ml of 50, 20, 10, 5, or 1 ppm DBP or TBP solutions were designed. A Petri dish containing only 5-μm-filtered seawater with approximately 20 eight-armed larvae was used as a positive control. As a negative control (blank), 5-μm-filtered seawater containing 0.4 ml of 99.5 vol% ethanol with approximately 20 eight-armed larvae was used. Each treatment consisted of three replicates. Each treated Petri dish was placed in a dark room at 20°C. After 24 h, the numbers of swimming, fallen and dead larvae were counted using a stereomicroscope under vertical illumination directly from above. Fallen larvae were confirmed as viable from movement of rods and cilia.

**Inhibition of metamorphosis**

The effects of DBP and TBP on larval metamorphosis were tested in the presence of dibromomethane (DBM, CH₂Br₂) as a chemical inducer. Metamorphosis experiments were conducted using a glass vessel, as reported by Agatsuma et al. (2006). The vessel consisted of a filter holder (SUS316, Shibata Scientific Technology, Tokyo, Japan) cut into upper and lower parts. The two parts were joined with a clamp and held upright in an acrylic stand. Dibromomethane (Wako Pure Chemical Industries) was added to the lower vessel, which was then sealed with a silicone rubber plug, and allowed to diffuse through a hydrophobic PTFE membrane (Advantec polymer, 47 mm
diameter, 0.2-μm pore size, Toyo Roshi Kaisha, Tokyo, Japan). Larvae added to the upper vessel were then exposed to DBM diffused from the lower vessel.

To obtain a one-half diluted solution of saturated DBM, which induces the highest metamorphic rate in *S. intermedius* larvae (Agatsuma et al. 2006), 250 ml of 5-μm-filtered seawater was added to a blacked-out 500-ml Erlenmeyer flask and plugged with a glass stopper. Twenty-five grams of DBM was then added and dissolved by stirring for 24 h. Seawater with insoluble DBM at the bottom was considered to be a saturated solution. Ten milliliters of a one-half diluted solution of saturated DBM was added to the lower vessel. Simultaneously, the upper vessel was clamped onto the lower one, and 20 ml of 5-μm-filtered seawater containing approximately 20 eight-armed larvae was added. Twenty-eight milliliters of 50, 20, 10, 5, or 1 ppm DBP or TBP solution was used, adding the same volume as described above for the survival experiment. An upper vessel containing only 28 ml of 5-μm-filtered seawater with approximately 20 eight-armed larvae was used as a positive control. Each treatment consisted of three replicates. Each treated glass vessel was placed in a dark room at 20°C. After 1 h, the DBM solution was removed by loosening the rubber plug. At this point, the DBM concentration in the upper vessel ranged from 52 to 66 ppm according to gas chromatograph-mass spectrometry analysis (Agatsuma et al. 2006). After 1, 2, 4, 8 and 24 h, the numbers of metamorphosed and dead larvae were counted using a stereomicroscope under vertical illumination directly from above. Metamorphosed individuals were defined as those whose larval arms had been absorbed, and which had a globular test, tube feet and spines.

**Statistical analysis**

The significance of differences in percentage larval metamorphosis among the treatments and times was analyzed by two-way repeated-measure analysis of variance (ANOVA) followed by Scheffé’s multiple comparison test. All the data showed a normal distribution and homogenous variance according to the Kolmogorov-Smirnov test and Levene’s test, respectively.

**Results**

**Survival of eight-armed larvae**

The percentage of swimming, fallen, and dead eight-armed larvae in filtered seawater containing different concentrations of 2,4-dibromophenol and 2,4,6-tribromophenol is shown in Table 1. In the presence of DBP solution, 93% of larvae kept swimming and 7% were knocked down at 1 ppm, 99-100% of larvae were knocked down at 5 and 10 ppm, and all died at 20 and 50 ppm. In the presence of TBP solution, all the larvae were observed swimming at 1 ppm, 99-100% were knocked down at 5 and 10 ppm, and 98-100% died at 20 and 50 ppm. All the larvae continued to swim under positive control and blank conditions, suggesting that 0.4 ml of ethanol in 28 ml of filtered seawater had no negative effect on the larvae.

**Table 1.** Percentage of swimming, fallen, and dead eight-armed larvae of *Strongylocentrotus intermedius* in filtered seawater with different concentrations of 2,4-dibromophenol and 2,4,6-tribromophenol dissolved in ethanol at the minimum concentration of 99.5 vol% after 24 h

<table>
<thead>
<tr>
<th>Bromophenol</th>
<th>Larval condition</th>
<th>Concentration (ppm)</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
<th>Control</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-dibromophenol</td>
<td>Swimming</td>
<td>93±8</td>
<td>1±2</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>100±0</td>
<td>100±0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fallen</td>
<td>7±8</td>
<td>99±2</td>
<td>100±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td>Dead</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>100±0</td>
<td>100±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>2,4,6-tribromophenol</td>
<td>Swimming</td>
<td>100±0</td>
<td>1±2</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>100±0</td>
<td>100±0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fallen</td>
<td>0±0</td>
<td>99±2</td>
<td>100±0</td>
<td>2±3</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
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<tr>
<td></td>
<td>Dead</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>98±3</td>
<td>100±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
</tbody>
</table>

*a* Filtered seawater.

*b* Filtered seawater with 0.4 ml of ethanol.

*n* = 3 for each treatment.
Inhibition of metamorphosis

Eight-armed larvae in filtered seawater containing different concentrations of DBP and TBP were exposed to a one-half dilution of saturated DBM solution for 1 h, and the percentage metamorphosis and survival rates over time are shown in Figures 1 and 2. Significant differences in percentage metamorphosis were found among concentrations and exposure times ($P < 0.0001$, Table 2). Under control conditions, the proportion of metamorphosed larvae rose markedly to 43% after 1 h, and then increased gradually to 75% after 24 h. The proportion of metamorphosed larvae at 1 and 5 ppm DBP solutions rose to 29% and 43%, respectively, after 1 h, and then increased sharply to 70% ($P < 0.05$) and gradually to 67%, respectively, after 24 h, which was not significantly different from that in the control at each time point ($P > 0.05$). In contrast, the proportion of metamorphosed larvae in the presence of 10 ppm DBP reached 25% after 1 h, and then increased slightly to 34% after 24 h, which was significantly lower than that in the control after 4-24 h ($P < 0.05$).

Table 2. Two-way repeated-measure analysis of variance (ANOVA) of percentage metamorphosis of Strongylocentrotus intermedius exposed to filtered seawater containing different concentrations of 2,4-dibromophenol and 2,4,6-tribromophenol in the presence of a one-half diluted solution of saturated DBM for 1 h

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
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<tr>
<td>2, 4-dibromophenol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>5</td>
<td>3665.997</td>
<td>61.100</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Concentration</td>
<td>5</td>
<td>8126.402</td>
<td>135.441</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Time × Concentration</td>
<td>25</td>
<td>478.026</td>
<td>7.967</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Error</td>
<td>72</td>
<td>60.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2, 4, 6-tribromophenol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>5</td>
<td>3349.165</td>
<td>29.415</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Concentration</td>
<td>5</td>
<td>6353.417</td>
<td>55.800</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Time × Concentration</td>
<td>25</td>
<td>341.951</td>
<td>3.003</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Error</td>
<td>72</td>
<td>113.860</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Percentage metamorphosis and survival rates of eight-armed larvae of Strongylocentrotus intermedius in filtered seawater containing 2,4-dibromophenol at 1, 5, 10, 20 and 50 ppm, and a filtered seawater control. Larvae were exposed to a one-half dilution of saturated dibromomethane solution for 1 h. Bars indicate standard deviation. Different letters indicate significance level at $P < 0.05$ among treatments at each time point. Asterisks indicate significance level at $P < 0.05$ between percentage metamorphosis after 1 h and those after 2, 4, 8, and 24 h. $n=3$ for each treatment.

Fig. 2. Percentage metamorphosis and survival rates of eight-armed larvae of Strongylocentrotus intermedius in filtered seawater containing 2,4,6-tribromophenol at 1, 5, 10, 20 and 50 ppm, and a filtered seawater control. Larvae were exposed to a one-half dilution of saturated dibromomethane solution for 1 h. Bars indicate standard deviation. Different letters indicate significance level at $P < 0.05$ among treatments at each time point. Asterisks indicate significance level at $P < 0.05$ between percentage metamorphosis after 1 h and those after 2, 4, 8, and 24 h. $n=3$ for each treatment.
All larvae exposed to 20 and 50 ppm DBP died after 1 h.

The proportion of metamorphosed larvae in the presence of 1 and 5 ppm TBP rose to 31\% and 36\%, respectively, after 1 h, and then increased markedly to 65\% \((P < 0.05)\) and gradually to 52\%, respectively, after 24 h, which was not significantly different from that in the control at each time point \((P > 0.05)\). In contrast, the proportion of metamorphosed larvae at 20 ppm TBP ranged from 4\% to 9\% within 24 h, which was significantly lower than that in the control after 8-24 h \((P < 0.05)\). The proportion of metamorphosed larvae at 20 ppm TBP increased markedly to 65\% after 1 h, and then increased slightly to 34\% after 24 h, which was significantly lower than that in the control at each time point \((P < 0.05)\). All larvae exposed to 50 ppm TBP died after 1 h.

**Discussion**

Almost all larvae were knocked down at 5-10 ppm, and died at 20-50 ppm after 24 h for both DBP and TBP, suggesting that DBP and TBP have strong toxicity for *S. intermedius* larvae. A one-half diluted solution of saturated DBM induced a metamorphosis rate of only 75\% in *S. intermedius* after 24 h, in contrast to a 100\% metamorphosis rate after 2 h \((Agatsuma et al. 2006)\). The difference in metamorphosis rate induced by DBM may be due to contamination with larvae that lack competency for metamorphosis. Nevertheless, DBP and TBP markedly reduced the rate of metamorphosis at 10 ppm and 10-20 ppm, respectively, suggesting that DBP and TBP have a strong inhibitory effect on the metamorphosis of *S. intermedius* larvae.

The present study demonstrated that DBP and TBP inhibit survival and metamorphosis of larvae of the sea urchin *S. intermedius*. DBP and TBP also inhibit survival and metamorphosis of larvae of the sea urchin *S. nudus* \((Agatsuma et al. 2008)\) and the Japanese abalone *Haliotis discus hannai* \((Li et al. 2009)\). These findings indicate that the two bromophenols may play a major role in the chemical defense of kelps against sea urchin and abalone recruitment in the field, although release of the bromophenols from other kelp species and their effective concentrations in the sea remain unknown. An ecologically relevant cue must be present at an effective concentration. Swanson et al. \((2006)\) reported that seawater collected in situ adjacent to the foliose red alga *Delisea pulchra* contained a low concentration of dissolved histamine below 5 nM and induced larval settlement of the sea urchin *Holopneustes purpurascens*. Histamine is therefore an ecologically relevant settlement cue. Detection of the release of DBP and TBP from other *Laminaria*, *Eisenia* and *Ecklonia* species, measurement of DBP and TBP concentrations in seawater adjacent to the kelps, and testing of the inhibitory effect of the water on settlement and survival of sea urchin larvae would shed more light on the ecological relevance of the two bromophenols.

DBP and TBP have a lower inhibitory activity on *S. intermedius* larvae than on *S. nudus* larvae, whose larval metamorphosis is strongly inhibited at 1 ppm \((Agatsuma et al. 2008)\). The sea urchin *S. nudus* is found on intertidal and subtidal rocky bottoms in the Pacific Ocean from Sagami Bay to Cape Erimo, Hokkaido, and in the Sea of Japan from Omi Island, Yamaguchi, to Cape Soya, northern Hokkaido \((Shigei 1995)\). The distribution of *S. nudus* overlaps greatly with that of the kelps *Eisenia bicyclis* \((Kawashima 1993)\). Agatsuma et al. \((2007)\) reported that feeding-deterrent metabolites extracted from the brown algae *Padina crassa*, *P. australis*, and *P. japonica* are more active on the sympatric snail *Chlorostoma lischkei* than on the allopatric snails *Omphalius rusticus* and *O. pfeifferi*. The higher inhibitory activity on *S. nudus* larvae suggests that DBP and TBP released from *Eisenia bicyclis* and *Ecklonia kurome* \((Shibata et al. 2006)\) may inhibit survival and metamorphosis of the sympatric *S. nudus* larvae rather than the allopatric *S. intermedius* larvae.

Ecklonia cava, and Ecklonia kurome (Taniguchi et al. 1992b) – produce phlorotannins, which deter feeding by H. discus hannai. Strongylocentrotus nudus grazes only on drifting thalli of E. bicyclis from which the phlorotannins have leached (Taniguchi et al. 1992a). Additionally, DBP and TBP released from E. bicyclis and E. kurome inhibit the settlement and survival of S. nudus (Agatsuma et al. 2008), S. intermedius, and H. discus hannai larvae (Li et al. 2009). These findings suggest that Eisenia and Ecklonia forests may use a double chemical defense mechanism – settlement and survival deterrents and feeding deterrents – to protect their populations from recruitment and grazing of sea urchins and abalones. Agatsuma et al. (2007) have reported that the mobile grazers H. discus hannai and H. discus discus are more sensitive than the sedentary grazer H. gigantea, and that the sympatric snail Chlorostoma lischkei is more sensitive than the allopatric snails Omphalius rusticus and O. pfeifferi, to feeding-deterrent metabolites extracted from the brown algae Padina crassa, P. australis, and P. japonica. Hence, further investigation of the degree of deterrence by DBP and TBP against larvae of the main species of sea urchins (Hemicentrotus pulcherrimus, Pseudocentrotus depressus, Anthocidaris crassispina) and abalone (H. discus discus, H. madaka, H. gigantea) living in southern Japan sympatric with Eisenia and Ecklonia forests would increase our understanding of the ecological relevance of these two bromophenols and the interactions between kelp forests and herbivores.

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References


Inhibition of Sea Urchin Larvae


2,4ジプロモフェノールと2,4,6トリプロモフェノールによるエゾババンウニ幼生の生残と変態の阻害

李 景玉・遠藤 光・吾妻行雄・谷口和也

褐藻アラメとクロメから抽出される2,4ジプロモフェノール（DBP）と2,4,6トリプロモフェノール（TBP）のエゾババンウニ幼生の生残と変態に及ぼす阻害効果を調べた。ほぼすべての幼生は DBP と TBP の 5 ppm と 10 ppm で落下し、20 ppm と 50 ppm で死亡した。ジプロモメタンを被曝させた対照区で幼生は 1 時間後に 43%，24 時間後に 75%が変態した。しかし、変態率は DBP の 10 ppm で25-34%，TBP の 10 ppm, 20 ppm で24-34%，4-10%と著しく低下した。いずれも 20 ppm と 50 ppm で 1 時間以内にすべての幼生が死亡した。海中水は DBP と TBP によってウニの加熱を化学的に防御しているのかもしれません。