Toxicity Test for Invert Soap against
Ciliate \textit{Colpoda aspera}

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Abstract An acute toxicity test for invert soap against \textit{Colpoda aspera} was performed by using monoxenic culture of \textit{C. aspera} isolated from activated sludge and \textit{Alcaligenes faecalis} IFO 13111. Four kinds of invert soap were used and those were as follows: benzenethonium chloride (BTC), benzalkonium chloride (BKC), didecyl dimethyl ammonium chloride (DAC) and an equally mixed preparation of methyl dodecyl xylilene benzyl ammonium chloride, methyl dodecyl xylilene trimethyl ammonium chloride and polyoxyethylene octyl phenyl ether (MAC**). Evaluation of the toxicity was based on the concentration of invert soap at which the specific growth rate of \textit{C. aspera} was reduced to 50% at 24 hours after the exposure to invert soap (EC50). EC50 of BTC, BKC, DAC and MAC** were 4.31, 2.89, 1.67 and 3.84 mg/l respectively. It was presumed that in actual application, BTC, BKC, DAC and MAC** should be diluted more than 66-, 98-, 47- and 40-fold respectively to reduce the concentrations below EC50. It was found that DAC produced the highest toxicity against \textit{C. aspera} and it was followed by BKC, MAC** and BTC in the order mentioned. On assessing the safety from the actually applicable concentrations and EC50, the highest safety was demonstrated by MAC** followed by DAC, BTC and BKC.


Key words: \textit{Colpoda aspera}, disinfectant, invert soap, toxicity

A variety of disinfectants are used for hygienic management of animal barns and slaughter houses. It is feared that inflow of disinfectants into biological waste water treatment processes and public water bodies may endanger ecological systems. In our previous studies of the influence of various disinfectants on activated sludge, ciliate protozoa were found highly susceptible to the disinfectants tested9-10. Ciliates are known to play an important role in soil and water. These organisms can be a useful indicator of operating conditions in activated sludge process11-14, and they are also helpful for cleaning of water by predation of harmful bacteria in water15-21. It is, therefore, necessary to test the toxicity of disinfectants to ciliates.

The following problems are involved in carrying out toxicity tests against ciliates. These include the choice of ciliate species, isolation, culture and preservation of ciliates, technique of acute toxicity test and establishment of a method for toxicity assessment15. The acute toxicity of aquatic organisms against pollu-
tants is usually estimated on the basis of the median tolerance limit (TL<sub>m</sub>) or median lethal concentration (LC<sub>50</sub>). Assessment by TL<sub>m</sub> and LC<sub>50</sub> is used for organisms such as fish, cladocerans and rotatorians having more than 2 days of mean generation time<sup>5,7</sup>). However, these indices are not applicable to ciliates having only a few hours of generation time<sup>7</sup>). SUDO et al. <sup>21</sup>) have determined the concentration of heavy metal at which the specific growth rate of ciliates was reduced to 50% as the median inhibitory limit (IL<sub>m</sub>) and assessed the toxicity from the value of IL<sub>m</sub>. IL<sub>m</sub> is based on exactly the same concept as the median effective concentration (EC<sub>50</sub>) which INAMORI et al. <sup>7</sup>) have used in assessment of the effects of synthetic detergents and soap. Only the terminology differs between IL<sub>m</sub> and EC<sub>50</sub>. PERSOONE et al. <sup>15</sup>) and SCHREIBER et al. <sup>19</sup>) have advocated the use of percentage at which the number of generation (NG) was inhibited. IL<sub>m</sub>, EC<sub>50</sub> and NG serve only the same purpose to obtain the same result.

In this study, an acute toxicity test of four kinds of invert soap to ciliates was carried out using EC<sub>50</sub> as an index. Because genus Colpoda are known to form a strong cyst in a dry or high-temperature or low-temperature environment<sup>25</sup>), Colpoda aspera, a species easy to maintain and control, was isolated from activated sludge and used. Enterobacter aerogenes or Escherichia coli has been used as a feed for genus Colpoda<sup>6,11,13,16,24</sup>). However, since this experiment was designed to evaluate the influence of ciliates on ecological system in natural water bodies, an aquatic bacterial speices, Alcaligenes faecalis was used as a feed for Colpoda aspera.

**Materials and Methods**

**Disinfectants**:

Hyamine solution (Sankyo Co., Ltd.), benzalkonium chloride (Wako Pure Chemical Industries, Ltd.), Astop (Eisai Co., Ltd.) and Pacoma (Eisai Co., Ltd.) were used in this test. The Hyamine solution was a preparation of 10% benzethonium chloride (BTC) solution. Benzalkonium chloride (BKC) was also a 10% solution. Astop was a 10% didecyl dimethyl ammonium chloride (DAC) solution. Pacoma was an invert soap solution composed of the mixture of 5% methyl dodecyl xylilene benzyl ammonium chloride and 5% methyl dodecyl xylilene trimethyl ammonium chloride, and further contained 5% nonionic surfactant, polyoxyethylene octyl phenyl ether (MAC<sup>+</sup>).

**Microorganisms**:

Ciliate Colpoda aspera (C. aspera) was isolated from the activated sludge of swine waste water treatment at our laboratory. Monoxenic culture with Alcaligenes faecalis IFO 13111 (A. faecalis) was carried out. An egg yolk-lettuce extract medium (EL medium) was used for the isolation, preservation and monoxenic culture of C. aspera and A. faecalis<sup>3,12,20</sup>). The EL medium (pH 6.8–7.0) was prepared by the following method. Lettuce leaves were dried at 105°C for 5 hours. Zero point three gram of dried lettuce leaves were suspended in 100ml of distilled water, boiled for 30 minutes and filtered through cotton gauze (solution A). Thereafter, 0.3 g of boiled egg yolk was suspended in 100 ml of distilled water, boiled for 30 minutes and filtered through cotton gauze (solution B). Solution A was mixed with equal volume of solution B and autoclaved at 121°C for 15 minutes.

Tripticase soy-broth and -agar media (BBL) were used for preservation and growth culture of A. faecalis. Preculture of A. faecalis using monoxenic culture with C. aspera was carried out in the EL medium.

Identification of the ciliates isolated was based on the classification of BURT<sup>2</sup>), using morphological characteristics including the silver line system.

**Growth experiment of microorganisms**:

Axenic culture of A. faecalis in the EL medium was continued at 25°C for 11 days. The number of bacterial cells was counted.
everyday. Monoxenic culture of \textit{C. aspera} and \textit{A. faecalis} in the EL medium was carried out at 25°C for 36 hours. The number of \textit{C. aspera} was counted at 3, 12, 18, 24, 30 and 36 hours after culture. In this monoxenic culture, the concentration of \textit{C. aspera} was adjusted to 2,880 cell/ml with two different concentrations of \textit{A. faecalis} adjusted to $3.5 \times 10^7$ and $7.9 \times 10^7$ cell/ml. \textit{C. aspera} at their logarithmic growth phase were used in this study.

Toxicity test:

The minimum inhibitory concentrations (MIC) of the invert soaps against \textit{A. faecalis} were determined by the following method. After dilution of the invert soap in the EL medium, \textit{A. faecalis} was inoculated and incubated at 25°C for 24 hours. It was then inoculated on sensitivity disk agar (Eiken Chemical Co., Ltd.). Proliferation of \textit{A. faecalis} after 24 hours of incubation at 35°C was examined.

The toxicity test against \textit{C. aspera} was carried out basically by the method of SUDO et al. and INAMORI et al., using monoxenic culture of \textit{C. aspera} and \textit{A. faecalis}. At first, axenic culture of \textit{A. faecalis} was carried out using the EL medium (25°C for 72 hours). At the same time, monoxenic culture of \textit{C. aspera} and \textit{A. faecalis} was carried out (25°C for 15±3 hours). These axenic and monoxenic culture solutions were mired to contain $5-8 \times 10^7$ cell/ml concentration of \textit{A. faecalis} and 720 cell/ml concentration of \textit{C. aspera}. Then, 1 ml of the above mixed solution was treated with 1 ml of the invert soap solution diluted with the EL medium and incubated at 25°C for 24 hours. The number of \textit{C. aspera} was counted microscopically. This test was repeated 6 to 10 times.

The concentrations of the invert soaps used in this experiment were decided by those of the principal ingredient. The Hyamine solution was the 100,000 mg/l solution of BTC. BKC and DAC were also the 100,000 mg/l solutions. MAC" was the 15\% mixture of the 3 ingredients and hence was the 150,000 mg/l solution.

Median effective concentration:

The median effective concentration (EC$_{50}$) was calculated as follows: At first, the specific growth rate ($\mu$) was obtained by the following equation.

$$\mu = \frac{(\ln N - \ln N_0)}{t} \quad \text{(1)}$$

where $\mu$ is the specific growth rate (day$^{-1}$), $N_0$; the number (cell/ml) of \textit{C. aspera} before the start of culture, $N$; the number (cell/ml) of \textit{C. aspera} at 24 hours after culture, and $t$; the time (day).

The percentage of residual $\mu$ was obtained by the following equation.

$$Y = \left(\frac{\mu_{tox}}{\mu_{cont}}\right) \times 100 \quad \text{(2)}$$

where $Y$ is the percentage of residual $\mu$, $\mu_{tox}$; the specific growth rate in the presence of the invert soap, and $\mu_{cont}$; the specific growth rate in the absence (controls) of the invert soaps.

In determination of EC$_{50}$, a logarithmic regression equation ($Y = a + b \cdot \ln X$) was first derived from $Y_1$ and $Y_2$% between which the 50\% line was interposed and from the logarithms ($\ln X_1$ and $\ln X_2$) of the corresponding concentrations of the invert soaps. Then $X$ mg/l was calculated when $Y = 50\%$. EC$_{50}$ was likewise determined from $X$ mg/l when $Y = 90\%$.

The generation time (hr) was also calculated by the following equation for comparison of the growth of \textit{C. aspera}.

$$t_g = \frac{(\ln 2/\mu)}{24} \quad \text{(3)}$$

where $t_g$ is the generation time and $\mu$; the specific growth rate calculated by Eq. (1).

Results

Growth of \textit{A. faecalis} at 25°C in EL medium:

As shown in Fig. 1, the highest specific growth rate was demonstrated by Day 3 (3.4 days$^{-1}$) and was observed by Day 7 (10$^8$ cell/ml). It was slightly slackened from Day 8 onwards.

Growth of \textit{C. aspera} at 25°C in monoxenic culture:

The growth curves of \textit{C. aspera} are shown in
Fig. 1. Growth of *A. faecalis* at 25°C in EL medium

Fig. 2. Its growth of *C. aspera* was more active in monoxenic culture with 7.9 × 10⁷ cell/ml of *A. faecalis* than with 3.5 × 10⁷ cell/ml. The number of *C. aspera* peaked 18 hours, but the growth was rapidly decreased subsequently and the generation time was also remarkably prolonged.

EC₁₀ and EC₅₀ against *C. aspera* and MIC against *A. faecalis*:

Table 1 shows EC₁₀ and EC₅₀ of the invert soaps against *C. aspera* and MIC against *A. faecalis*. EC₁₀ of BTC, BKC, DAC and MAC⁺⁺ against *C. aspera* were 1.04, 0.92, 0.50 and 1.41 mg/l respectively. EC₅₀ were 4.31, 2.89, 1.67 and 3.84 mg/l respectively. MIC of these disinfectants against *A. faecalis* were 150, 150, 75 and 200 mg/l respectively. The average initial bacterial count in this experiment was 5.5 × 10⁷ cell/ml. The specific growth rate and generation time of *C. aspera* at 24 hours in the control culture averaged 2.48 day⁻¹ and 6.94 hours respectively.

Relation between normal concentration for actual application to disinfection of animal barns and EC₅₀:

Table 2 shows the relation between normal concentration of these invert soaps for application to disinfection and EC₅₀ against *C. aspera*. The mean normal concentrations of BTC, BKC, DAC and MAC⁺⁺ were estimated to be 285, 285, 80 and 150 mg/l respectively. When these values were divided by EC₅₀, more than 66-, 98-, 47- and 40-fold dilutions were considered necessary for BTC, BKC, DAC and MAC⁺⁺ respectively in actual application to control the growth inhibition of *C. aspera* not to exceed 50%.

**Discussion**

*A. faecalis* has been reported as a prey bacterium for predation by *Vorticella microstoma*²² and *Colpidium campylum*²³. The predation of *A. faecalis* by *C. aspera* has not been described earlier. In the present experiment of axenic culture of *A. faecalis* and
Toxicity Test for Invert Soap against *C. aspera*

**Table 1.** EC<sub>10</sub> and EC<sub>50</sub> of the invert soaps against *C. aspera* and MIC against *A. faecalis*

<table>
<thead>
<tr>
<th>Invert soap</th>
<th>EC&lt;sub&gt;10&lt;/sub&gt; (mg/l)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (mg/l)</th>
<th>MIC (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzethonium chloride (BTC)</td>
<td>1.04±0.19*</td>
<td>4.31±0.48</td>
<td>150</td>
</tr>
<tr>
<td>Benzenalkonium chloride (BKC)</td>
<td>0.92±0.33</td>
<td>2.89±0.84</td>
<td>150</td>
</tr>
<tr>
<td>Didecyl dimethyl ammonium chloride (DAC)</td>
<td>0.50±0.15</td>
<td>1.67±0.52</td>
<td>75</td>
</tr>
<tr>
<td>Methyl dodecyl xylenbenzyl ammonium chloride+ α (MAC**)</td>
<td>1.41±0.15</td>
<td>3.84±0.36</td>
<td>200</td>
</tr>
</tbody>
</table>

*Mean±SD.*

Significant differences (p<0.01) in EC<sub>10</sub> are noted among the invert soaps except between BTC and BKC. Significant differences (p<0.01 or 0.05) in EC<sub>50</sub> are noted except between BTC and MAC**

**Table 2.** Relation between normal applicable concentrations (NC) and EC<sub>50</sub> of the four invert soaps

<table>
<thead>
<tr>
<th>NC</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Dilution</th>
<th>NC/EC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTC</td>
<td>285</td>
<td>4.3</td>
<td>&gt;66</td>
</tr>
<tr>
<td>BKC</td>
<td>285</td>
<td>2.9</td>
<td>&gt;96</td>
</tr>
<tr>
<td>DAC</td>
<td>90</td>
<td>1.7</td>
<td>&gt;47</td>
</tr>
<tr>
<td>MAC**</td>
<td>150</td>
<td>3.8</td>
<td>&gt;40</td>
</tr>
</tbody>
</table>

* Dilution required to keep the concentration below EC<sub>50</sub>

monoxenic culture of *A. faecalis* and *C. aspera* at 25°C in the EL medium, these microorganisms were both satisfactorily proliferated (Fig. 1 and 2). This experiment indicated that *A. faecalis* could be used as a prey for *C. aspera* and that these microorganisms could proliferate by monoxenic culture in the EL medium. Sorensen's solution<sup>11</sup>, phosphate buffer<sup>16, 21</sup>, Neff's amoeba saline<sup>19</sup>, cerophyl medium<sup>18</sup> or EL medium<sup>2, 12, 20</sup> has been used for monoxenic culture of ciliates and bacteria. In culture with first-mentioned 3 media, only ciliates are proliferated but bacteria are not. In culture with the latter 2 media, both ciliates and bacteria are proliferated. The viability and proliferation of ciliates can be maintained longer in the growth medium for both ciliates and bacteria (such as EL medium) than that for only ciliates. This seems to indicate that the use of EL or cerophyl medium is more advantageous in undertaking a toxicity test against ciliates, because these media exert only a little adverse effect against bacterial growth.

On examining EC<sub>50</sub> of the 4 invert soaps used against *C. aspera*, the lowest value was demonstrated by DAC (1.67 mg/l), followed by BKC (2.89 mg/l), MAC** (3.84 mg/l) and BTC (4.31 mg/l). EC<sub>10</sub> of DAC was also lowest (0.5 mg/l), followed by those of BKC (0.92 mg/l), BTC (1.04 mg/l) and MAC** (1.41 mg/l). MIC of DAC, BKC, BTC and MAC** against *A. faecalis* were 75, 150, 150 and 200 mg/l respectively. These result showed that *C. aspera* and *A. faecalis* were most susceptible to DAC. Large differences between MIC against *A. faecalis* and EC<sub>10</sub> or EC<sub>50</sub> against *C. aspera* demonstrated that EC<sub>10</sub> and EC<sub>50</sub> were less than MIC.

We have previously investigated the inhibitory concentrations of these 4 disinfectants against the activities of microorganisms in activated sludge and found that the inhibitory concentrations of BTC, BKC, DAC and MAC** were 5, 5, 2 and 5 mg/l respectively<sup>9</sup>. These values were close to EC<sub>50</sub> of these disinfectants against *C. aspera*. This may suggest that inhibition of the activity of microorganisms in activated sludge by exposure to these disinfectants was caused primarily by damage to
ciliates existing in activated sludge.

For actual application of BTC, BKC, DAC and MAC\textsuperscript{a} to disinfection of animal barns, it is presumed that these disinfectants should be diluted more than 66-, 98-, 47- and 40-fold respectively to reduce normal application concentrations below EC\textsubscript{50}. This may show that MAC\textsuperscript{a} is the safest disinfectant while BKC is most unsuitable for application to biological treatment of swine waste water.

This study demonstrated that the susceptibility of \textit{C. aspera} was highest to DAC and was second highest BKC. On evaluating the safety of these soaps in relation to the normal application concentrations and EC\textsubscript{50}, the highest safety was demonstrated by MAC\textsuperscript{a} and the safety was reduced in the order of DAC, BTC and BKC. The relations between temperature and the growth of \textit{C. aspera} and among temperature, time and EC\textsubscript{50} are still unclear. The toxicity of various kinds of disinfectants to other ciliate species should also be studied.

**Acknowledgement**

The authors sincerely wish to thank to Dr. S. IMAI, Department of Veterinary Parasitology in our university, for identifying ciliate protozoa.

**References**


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逆性石鹸の纖毛虫 Colpoda aspera に対する毒性試験

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活性汚泥由来の Colpoda aspera は Alcaligines faecalis IFO 13111 と共に培養され、それに 4 種の逆性石鹸を加えることによって C. aspera に対する急性毒性試験を試みた。逆性石鹸としては、benzethonium chloride (BTC), benzalkonium chloride (BKC), didecyl dimethyl ammonium chloride (DAC), および methyl dodecyl xylilene benzyl ammonium chloride, methyl dodecyl xylilene trimethyl ammonium chloride と polyoxyethylene octyl phenyl ether の 3 種等量合剤 (MAC**) を供試した。毒性評価としては各逆性石鹸添加 24 時間後における C. aspera の増殖速度が 50% に低下する濃度 (EC50) を求めた。その結果，C. aspera に対する BTC, BKC, DAC および MAC** の EC50 はそれぞれ 4.31, 2.89, 1.67 および 3.84 mg/l であった。また、BTC, BKC, DAC および MAC** の実際の使用濃度は C. aspera に対する EC50 値以下とする必要があり、その希釈度はそれぞれ 66, 98, 47 および 40 倍以上であると推察された。
以上の結果から，C. aspera に対する毒性は DAC が最も強く，次いで BKC, MAC**, BTC の順であった。一方，実際の使用濃度と EC50 値から供試逆性石鹸の安全性を評価したところ，MAC** が最も安全で，次いで DAC, BTC, BKC の順であることが明らかとなった。

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