Toxicity of linear alkylbenzene sulphonate (LAS) to juvenile kuruma shrimp, Penaeus japonicus : a histopathological study on acute and sub-chronic levels

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TOXICITY OF LINEAR ALKYLBENZENE SULPHONATE (LAS) TO JUVENILE KURUMA SHRIMP, *PENAEUS JAPONICUS*: A HISTOPATHOLOGICAL STUDY ON ACUTE AND SUB-CHRONIC LEVELS*

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The toxicity and the histopathological changes induced by sodium *n*-dodecylbenzene sulfonate (C_{12}LAS) to juvenile kuruma shrimp (*Penaeus japonicus*) were examined during acute (96 hours) and sub-chronic exposure (14 days). Histological analysis revealed that C_{12}LAS affected only the gills during acute exposure. Epithelial cells of the gills became necrotic when exposed to the lethal concentration (12 mg/*l*). At the lowest concentration (0.75 mg/*l*), the distal parts of the gill filaments showed bulging and in most cases became atrophied. Gill and stomach alterations were common in juvenile kuruma shrimp exposed to the sub-chronic level (0.042 mg/*l*) for 14 days. The possible cause of surfactant-induced mortality is asphyxia.

Key words: Toxicity, LAS, Histopathology, Kuruma shrimp (*Penaeus japonicus*)

Introduction

The most frequently used surfactant in detergents is Linear Alkylbenzene Sulphonate (LAS). Consumption of LAS in U.S.A., Japan and Western Europe in 1987 was 984,000 metric tons (Lewis, 1991). This surfactant has been found in freshwaters (Mason, 1991), marine and coastal waters (Eganhouse *et al.*, 1983; Takada and Ishiwari, 1991), as well as in the sediment of aquaculture ponds (Chen *et al.*, 1992), and has potential to cause water pollution. Many studies about toxicity of LAS to fish (Swedmark *et al.*, 1971; Abel and Skidmore, 1975; Lal *et al.*,1983), aquatic invertebrates like *Daphnia magna* (Maki and Bishop, 1979; Lewis and Perry, 1981), and oyster (Granmo, 1972), among others, have been conducted. The effects of LAS most commonly monitored are those of behavioral (Swedmark *et al.*, 1971; Tatsukawa and Hidaka, 1978), physiological (Maki, 1979; Part *et al.*, 1985), and histological (Abel and Skidmore, 1975) natures. However, the majority of these studies employed freshwater animals, in particular fish, as test organism. Seldom marine and coastal organisms such as shrimps have been used (Lewis, 1991).

Penaeid shrimps like *Penaeus monodon* and *Penaeus japonicus* are used for human consumption, and are among the most important aquatic organisms cultured in coastal areas. Shrimp seeds are generally produced in hatcheries or semi-closed water bodies and released into ponds or coastal waters at the juvenile stage, where they grow until marketable size. In these environments, which generally have access to open waters, the shrimp have many chances of being exposed to several pollutants, including LAS. As mentioned above, however, very little information is available on the toxicity of LAS to shrimp.

Long term, sub-lethal or chronic toxicity tests are commonly designed to provide insight on the effect of various substances on the biological processes, including histological changes of tissues and cells, survival, growth, and reproduction success of an organism (Forlin *et al.*, 1986). This study was therefore conducted to determine possible toxic effects of LAS to juvenile kuruma shrimp (*Penaeus japonicus*) at the acute and sub-chronic levels, including histopathological alterations induced by the surfactant.

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Materials and Methods

Test organisms

Juvenile kuruma shrimp (*Penaeus japonicus*) at the stage of PL_{33} were obtained from Amaha Gyogyo Kyodokumiai Shubyoseisan Center, Japan on July, 1993. The shrimp were acclimatized to the laboratory conditions for 1 week. During acclimation, shrimp were kept in sea water and fed on *Artemia* nauplii and a commercial diet. Food was with held 1 day before the toxicity tests.

Preparation of test solutions

The surfactant used in this study was sodium *n*-dodecylbenzene sulfonate (C_{12}LAS) (WAKO, Japan). A 1,000 mg/*l* surfactant stock solution was prepared by dilution with deionized water. The resulting aqueous solution was then diluted with natural seawater to prepare the test solutions.

Natural seawater used to prepare test solutions was collected from Sagami Bay. The water was aerated strongly for 3–7 days to cause biodegradation of any surfactants which might have been present (Swisher, 1970), filtered twice in 5 μm and 0.45 μm-pore filters, and stored in polyethylene tanks until use. The water was aerated again for at least 1 day before use to ensure oxygen saturation.

Test Procedures

The acute toxicity test was conducted in duplicate using 1 *l* glass beakers as exposure chambers. Ten juvenile shrimp (321 ± 6 mg in weight and 2.5 ± 0.2 cm in total length) were placed in each chamber. The chambers and the juveniles were allotted following a Table of random numbers. The concentrations of C_{12}LAS used in this experiment were 0 mg/*l* (as a control), 0.75, 1.5, 3.0, 6.0, and 12.0 mg/*l*. Based on the result of a test of stability of C_{12}LAS in sea water conducted previously (Toshima, 1993 unpublished data), the type of test chosen was renewal test with 100% exchange of the test solutions per day. The duration of the test was 96 hours and during this period the water was not aerated and no feeding was provided. Mortality of the shrimp was monitored at 3, 6, and 12 hours after exposure and at 12-hour intervals thereafter until 96 hours. Shrimp which did not respond to stimulation at these periodical inspections and all shrimp alive at the end of the experiment (5–10 shrimps per group) were collected and fixed in Bouin's fixative for histological analysis. The sub-chronic test was conducted in duplicate in 2 *l* flow-through beakers with an exchange rate of 100% per day. The exposure concentration was 1% of the 96-hour LC_{50} value (4.2 mg/*l*) of juvenile kuruma shrimp determined in the previous experiment, or 0.042 mg/*l*. The shrimp were exposed to this concentration for 14 days, during which time they were fed a commercial diet and *Artemia* nauplii. Shrimp were sampled every 7 days (about 3 shrimps in each occasion) and processed for histology as described above. The range of pH, dissolved oxygen content, and water temperature of the test waters in the acute toxicity tests were 7.89–8.05, 4.2–6.5 mg/*l*, and 24.5–25.0℃, respectively, and in the sub-chronic tests 7.88–8.03, 5.1–6.4 mg/*l*, 24.9–27.0℃, respectively. In both tests, salinity was constant at 30 ppt. These conditions were within the levels that are suitable for post-larvae rearing (Kafuku and Ikenoue, 1983; Liao, 1992).

Histological procedures

Sampled shrimp were fixed in Bouin's solution for 1–2 days, washed and then stored in 70% ethyl alcohol until processing. Tissue blocks for histological examination were dehydrated in an ethanol series, cleared in toluene, and embedded in Paraplast. Tissue sections 5 μm thick were stained with Harris hematoxylin and eosin, according to standard procedures for light microscopy (Luna, 1968).

Statistical analysis

The log-probit distribution of percentage mortality versus concentration of C_{12}LAS, used to estimate the LC_{50} value, was calculated using the method of Finney (1971). The 95% confidence interval of the LC_{50} was calculated after Stephan (1977).

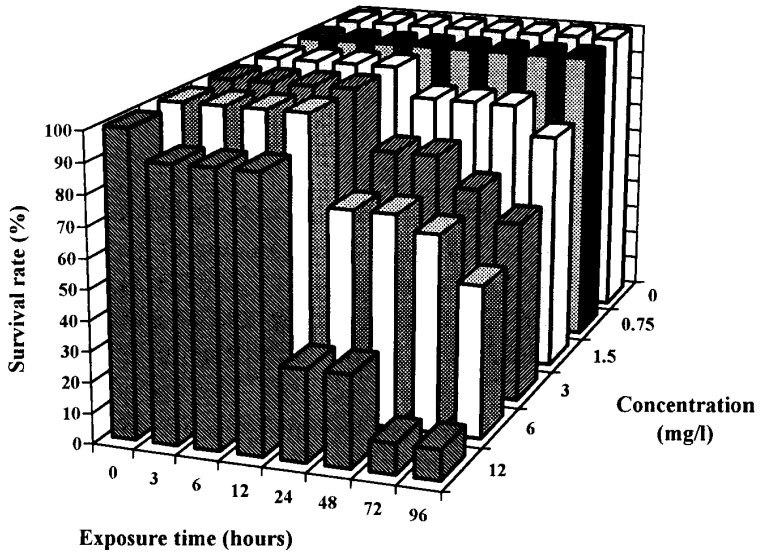


Fig. 1. Survival rate of *Penaeus japonicus* in acute toxicity test of LAS.

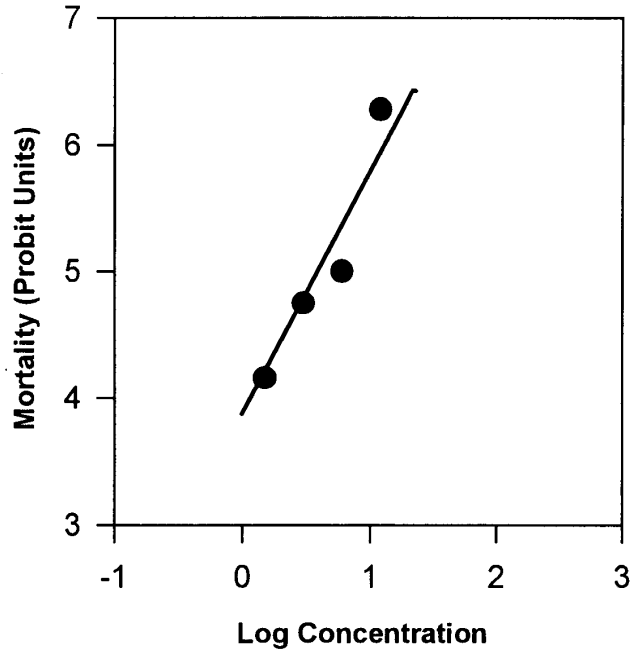


Fig. 2. Log–probit relationship between mortality of juvenile kuruma shrimp and log concentration of LAS at 96 hours acute toxicity test.

Results

LC_{50} determination

The survival rates of *Penaeus japonicus* exposed to different concentrations of C_{12}LAS during the acute toxicity test are presented in Fig. 1. Mortalities began faster and were higher with increasing concentrations of C_{12}LAS. Final mortalities were about 50% at 6.0 mg/*l* and 90% at 12.0 mg/*l*. The log–probit relationship between mortality of juvenile kuruma shrimp and log concentration of C_{12}LAS in the 96-hour acute toxicity test is presented in Fig. 2. From that equation ($y=3.693+2.095x$), the 96-hour LC_{50} of C_{12}LAS for *P. japonicus* (with 95% confidence interval) was estimated to be 4.2 (3.04–5.79) mg/*l*.

Histopathology at the acute level

In macroscopic appearance, the gills turned totally or partially black in about 50% of the shrimp in 6.0 mg/*l* and in 100% of the shrimp in 12.0 mg/*l* after 24 hours of exposure. In contrast, the gills of shrimp in 0.75, 1.5, and 3.0 mg/*l*, did not show any noticeable blackening.

The representative gill structure of the control shrimp and of shrimp exposed to 12.0 mg/*l* of C_{12}LAS are shown in Figs. 3 and 4, respectively. In the latter, the tips of the secondary gill filaments are fused and condensed and the epithelial cells are necrotic. The tips of the secondary gill filaments of shrimp exposed to 0.75 mg/*l* for 96 hours showed bulging (Fig. 5). In most cases, the gill filaments were atrophied. Similar changes were observed in the gills of shrimp exposed to 1.5 and 3.0 mg/*l*.

Histopathology at the sub-chronic level

Histopathological examination revealed major changes in the gills and stomach of shrimp exposed to the sub-chronic level (0.042 mg/*l*) of C_{12}LAS. The gills of shrimp exposed for 7 days showed bulging and necrosis in the tips of the secondary gill filaments (Fig. 6). Basal portions of the secondary filaments enlarged into bulb-like structures, and finally these bulges became the dominant structures in the secondary gill filaments of the shrimp after 14 days (Fig. 7).

The structure of the stomach epithelium of control shrimp is shown in Fig. 8. The stomach of shrimp exposed to 0.042 mg/*l* for 14 days showed dechromatization of the nuclei of epithelial cells and detachment of the columnar epithelia (Fig. 9). Cloudy swelling of the epithelial cells was also observed.

Fig. 3. Light micrograph of a gill filament from a control shrimp, showing the gill central axis, as well as the primary and secondary filaments. Cross section; bar represents 20 μm.

Fig. 4. Gill section of shrimp exposed to acute lethal concentration (12 mg/*l* of C_{12}LAS). The tips of secondary filaments are fused and condensed (arrow) and the epithelial cells are necrotic (head arrow). Longitudinal section; bar represents 30 μm.

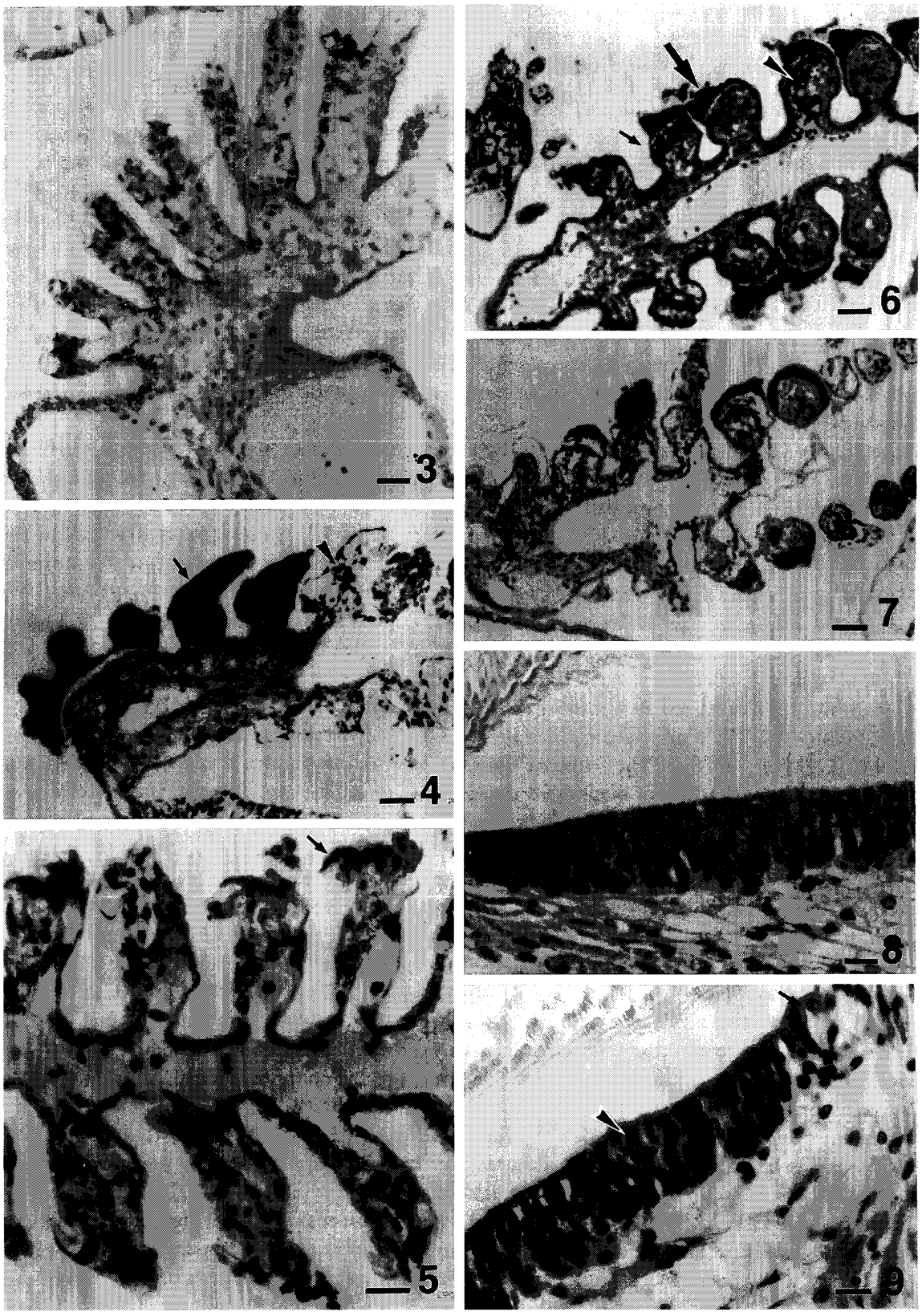
Fig. 5. Gill section of shrimp exposed to 0.75 mg/*l* of C_{12}LAS for 96 hours showing bulging of the tips of secondary filaments (arrow). Longitudinal section; bar represents 20 μm.

Fig. 6. Gill section of shrimp exposed to the sub-chronic level (0.042 mg/*l*) for 7 days, showing deposition of melanin pigments (large arrow), bulging (small arrow) and necrosis (head arrow) in the tips of secondary filaments. Longitudinal section; bar represents 40 μm.

Fig. 7. Gill section of shrimp exposed to the sub-chronic level (0.042 mg/*l*) for 14 days, showing bulging in the basal portion of the secondary filament. Longitudinal section; bar represents 40 μm.

Fig. 8. Section of the ventral part of the posterior chamber of the stomach of control shrimp, showing lumen and columnar epithelium. Cross section; bar represents 10 μm.

Fig. 9. Stomach section of shrimp exposed to the sub-chronic level (0.042 mg/*l*) for 14 days, showing dechromatization of the nuclei of epithelial cells (head arrow) and detachment of columnar epithelium (arrow). Cross section; bar represents 10 μm.



Figs. 3–9

Discussion

Shrimp in all concentrations of C_{12}LAS showed normal swimming behavior until 12 hours of exposure, but thereafter some individuals in 3.0, 6.0, and 12 mg/*l* of C_{12}LAS began to swim actively near the surface of the water. After 24 hours, about 40% of the shrimp in 6.0 and 12 mg/*l* swam sideways, followed by complete immobilization and death. These findings agree with the reported effects of surfactants on the locomotion of fish (Swedmark *et al.*, 1971; Olsen and Hoglund, 1985).

C_{12}LAS had a significant effect on the survival rate of the shrimp exposed to all concentrations above 1.5 mg/*l*. The reported LC_{50} values of LAS for aquatic invertebrates, regardless of exposure time, range from 0.7 to 92.0 mg/*l* (Table 1), but in most cases the type of LAS utilized was not reported. Toxicities of C_{12}LAS to invertebrates range from 2.7 mg/*l* for *Daphnia magna* (Lewis and Perry, 1981) to 19.4 mg/*l* for a gastropod (Hendricks *et al.*, 1974). Thus, the LC_{50} value of C_{12}LAS to kuruma shrimp PL_{33} estimated in this research (4.2 mg/*l*) falls in the lower range of those values and is similar to that of *D. magna*, one of the most sensitive aquatic organisms to chemicals, including LAS (see APHA, 1976).

The Japan Chemical Test Society (JCTS, 1987) reported that the LC_{50} values of LAS for *P. japonicus* were 0.960 mg/*l* for mysid stage, 1.43 mg/*l* for 1 day old (PL_1) post larvae, 1.80 mg/*l* for PL 14, and 6.24 mg/*l* for PL_{45}. The present study revealed that the LC_{50} of C_{12}LAS to *P. japonicus* in stage PL_{33} is 4.24 mg/*l*. Although the JCTS report did not specify the kind of LAS used, these results suggest that the toxicity of LAS to *P. japonicus* decreases with increasing developmental stage. This observation agrees with the pattern of toxicity of LAS to Mollusca (Granmo and Jorgensen, 1975), bivalve (Swedmark *et al.*, 1971, Bressan *et al.*, 1989), Echinodermata (Bressan *et al.*, 1989), and fish (Pickering and Tatcher, 1970; Hokanson and Smith, 1971; Swedmark *et al.*, 1971).

In macroscopic appearance, shrimp exposed to 6.0 and 12.0 mg/*l* showed blackening of the gills. This condition also occurred in shrimp exposed to cadmium (Couch, 1978; Nimmo *et al.*, 1977), copper (Lightner, 1977; William *et al.*, 1982), potassium permanganate, ammonia and nitrite (Sindermann and Lightner, 1988). The ultimate cause of blackening in this study was not determined. However, massive congestion of hemocytes in the gills, sometimes resulting in disfunction of the gill, has been noted by Lightner (1983). Moreover, deposition of melanin pigments at the hemolymph canal was often recognized in this study (Fig. 6).

Histologically, the surfactant provoked histopathological changes in the stomach and gills. Surfactant-induced histological changes in the digestive tract have been reported in other animals. Oberle *et al.* (1995) reported that surfactants caused damage in the mucosa of the jejunum and colon of rat. The intestine of bluegills exposed to 1.0 mg/*l* Linear Tridecyl Benzene (LTB) showed enlargement of blood vessels, while fish exposed to 1.15 mg/*l* LTB showed sloughing of intestinal cells (Dolan, 1974 in Maciorowski *et al.*, 1977). Maciorowski *et al.* (1977) found intestinal damage on fingernail clams exposed to 1 and 10 mg/*l* of Linear Tridecyl Benzene Sulfonate (LTBS) for 1 day. A generalized loss of cilia, cell vacuolation and detachment of the columnal epithelium were observed at 1.0 mg/*l* and a marked reduction in the size of the intestinal cells at 10 mg/*l*. We observed detachment of the columnal epithelium also in juvenile *P. japonicus* exposed to 0.042 mg/*l* of C_{12}LAS for 14 days. The dechromatization of the ephithelia observed in this study apparently has not been detected by previous studies on the toxicity of LAS to aquatic organisms.

Maciorowski *et al.* (1977) suggested that the feeding mechanism of fingernail clam provided a route of entry for LTBS into the intestinal epithelium. In contrast, it is likely that LAS may enter the digestive tract of *P. japonicus* through osmoregulatory rather than feeding processes. Both oral and anal drinking have been observed in a wide range of crustacean species; moreover, the gut has been characterized as a site of ion-dependent fluid absorption in both hypo- and hyper-regulating crusta-

Table 1. Reported acute toxicities of LAS to aquatic invertebrates.

Kind of LAS	Test species	LC_{50} (mg/l) (Exposure time)	Reference
LAS	*Gammarus pseudolimnaeus*	7.0 (96 hrs)	Arthur (1970)
LAS	*Physa integra*	9.0 (96 hrs)	〃
LAS	*Campeloma decisum*	27.0 (96 hrs)	〃
LAS	*Penaeus japonicus* (mysis)	0.96 (96 hrs)	JCTS (1987)
LAS	*Penaeus japonicus* (PL_1)	1.43 (96 hrs)	〃
LAS	*Penaeus japonicus* (PL_{14})	1.80 (96 hrs)	〃
LAS	*Penaeus japonicus* (PL_{45})	6.24 (96 hrs)	〃
$C_{11.8}$LAS	*Daphnia magna*	1.8–5.6 (48 hrs)	Lewis and Suprenant (1983)
$C_{11.8}$LAS	*Dero* sp.	1.7 (48 hrs)	〃
$C_{11.8}$LAS	*Dugesia* sp.	1.8 (48 hrs)	〃
$C_{11.8}$LAS	*Gammarus* sp.	3.3 (48 hrs)	〃
$C_{11.8}$LAS	*Paratanytarsus Parthenogenica*	23 (48 hrs)	〃
$C_{11.8}$LAS	*Rhabditis* sp.	16 (48 hrs)	〃
C_{12}LAS	*Daphnia magna*	3.5 (48 hrs)	Kimerle and Swisher (1977)
C_{12}LAS	*Daphnia magna*	5.9 (48 hrs)	Maki and Bishop (1979)
C_{12}LAS	*Daphnia magna*	2.7 (48 hrs)	Lewis and Perry (1981)
C_{12}LAS	*Goniobasis* sp.	19.4 (24 hrs)	Hendricks *et al.* (1974)
C_{12}LAS	*Isonychia* sp.	5.3 (96 hrs)	Dolan *et al.* (1974)
C_{13}LAS	*Goniobasis* sp.	92 (24 hrs)	Hendricks *et al.* (1974)
C_{14}LAS	*Daphnia magna*	0.7 (48 hrs)	Maki and Bishop (1979)

ceans (Mantel and Farmer, 1983). Thus, LAS may enter the digestive tract and probably be absorbed in the ephitelial cells of the gut, causing their breakdown. Another route of entry of LAS into the body may have been provided by osmoregulatory processes in the gills of the shrimp, since the gill is also a primary site of ion absorption (Mantel and Farmer, 1983).

It is generally assumed that surfactants first affect the respiratory structures in aquatic animals (Swedmark *et al.*, 1971; Abel, 1974). Effects on the gills of fish have been widely reported, including loss of mucosal cells from the gill lamina, hematomas, swelling or thickening of the gill epithelium, and acute inflammation of the gill filaments (Abel and Skidmore, 1975). In lethal exposure, C_{12}LAS caused extensive damage only to the gills of the juvenile kuruma shrimp, involving necrosis, fusion and condensation of the secondary gill filaments. Moreover, gill damage also occurred in the shrimp exposed to C_{12}LAS at the sub-chronic level. Singer *et al.* (1993) suggested that asphyxia was the immediate cause of death of kelp forest mysids exposed to complex mixtures of anionic and nonionic surfactants and solvents. These facts prompt us to suggest that the death of the shrimp exposed to high concentrations of C_{12}LAS in this study may have been caused primarily by loss of gill functions.

The capability of surfactants to interact with proteins and alter membrane permeability (Swisher, 1970) suggest that they act as general tissue poisons rather than a highly specific inhibitor (Maciorowsky *et al.*, 1977). The interaction of surfactants with proteins also indicates the likelihood of their widespread systemic toxic effects internally (Abel, 1974). The damage inflicted on the stomach and gills of the shrimp perhaps is the result of these characteristics.

In the present study, gill alterations were found in *P. japonicus* exposed to 0.042 mg/l of C_{12}LAS for 14 days in the sub-chronic test. A similar study conducted on fish has found also histopathological alterations, but at a much lower concentration of LAS. Misra *et al.* (1985) reported that the gills of fingerling *Cirrhina mrigala* exposed to 0.005 mg/l of LAS in a sub-chronic test showed damage of the

epithelium of the secondary lamellae. Moreover, the epithelial cells in the epidermis of the skin were found to secrete more mucus in fish exposed to sub-chronic levels of LAS than those of control fish (Misra *et al.*, 1987). These findings indicate a wide variation in susceptibility of marine organisms to LAS-induced pathologies. Meanwhile, the reported concentrations of LAS in natural marine environments also vary widely. For example, Kikuchi *et al.* (1986) found a range of 0.0008 to 0.03 mg/*l* of LAS in Tokyo Bay waters. The concentration of LAS in coastal waters of Spain ranged from 0 to 0.26 mg/*l* (Martinez *et al.*, 1989). Thus, while the potential for LAS-induced pathologies in natural environments exist, it is most probably a rare event conditioned to a spatial and temporal match of high concentrations of LAS and susceptible organism (conf. Kush and Petersen, 1997). In the case of kuruma shrimp, LAS-induced damage seems most likely to occur in shrimp culture ponds. For instance, Chen *et al.* (1992) reported a range of 0.044 to 4.317 mg/*l* in the water of shrimp culture ponds in South Taiwan. Further studies on LAS toxicity should address these possibilities and attempt to determine the no-effect concentration based on histological examination. Also, more detailed assessment of the LAS concentration in natural environments is needed, with special attention paid to the fate of LAS in water and soil (Huber, 1989).

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クルマエビ稚仔に及ぼすLASの毒性：急性・亜急性レベルでの病理組織学的研究

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クルマエビ稚仔に対するLAS（直鎖アルキルベンゼンスルホン酸）の急性（96時間暴露）ならびに亜急性（14時間暴露）毒性を，病理組織学的立場から検討した。その結果，急性症状として高濃度 (12 mg/*l*) で鰓上皮細胞の壊死，低濃度 (0.75 mg/*l*) で同細胞の浮腫・萎縮が観察された。また，低濃度 (0.042 mg/*l*) 亜急性毒性として鰓と消化管（特に胃）における病態が認められた。以上の結果から，LASはクルマエビ稚仔の鰓機能に損傷を与え，呼吸不全を引き起こすものと推察された。

キーワード：毒性，LAS（直鎖アルキルベンゼンスルホン酸），病理組織学的，クルマエビ