First incidence of loose-shell syndrome disease in the giant tiger shrimp *Peneaus monodon* from the brackish water ponds in Bangladesh

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**ABSTRACT**

The recent incidence of loose-shell syndrome disease (LSSD) in grow-out shrimp ponds appears to be a major problem in Bangladesh. Therefore, the aims of our study were (i) to observe clinical signs of LSSD to confirm its incidence and (ii) to identify the probable causative agents for LSSD in shrimp. Sampling was conducted randomly from five LSSD-affected ponds and three non-affected ponds near Bakkhali River; ecological parameters were measured. A total of 180 healthy shrimps were used for this experiment, where LSSD-affected shrimp extracts were either injected into the shrimps or mixed with water in the experimental tanks. Finally, microbial examinations were performed to identify the possible LSSD causative agents from the infected individuals. The total shrimp production was higher in ponds with healthy populations (185 kg/ha) than from LSSD-affected ponds (126–146 kg/ha); the survival rate of shrimp at harvest was significantly different \(p < 0.01\) between normal and LSSD-affected ponds. The prevalence of infection with white spot syndrome virus (WSSV) and monodon baculovirus (MBV) was found to be lower than that of the *Vibrio* infections. During the investigation, 8% of LSSD-affected individuals were WSSV positive and 5% were MBV positive, and 4% were infected by both. Our study suggested that the prevalence of LSSD in tiger shrimp might be associated with multiple *Vibrio* bacterial infections, poor soil and water quality, as well as poor pond management.

**Introduction**

Declines in shrimp production in grow-out ponds are mainly a result of several diseases caused by bacteria, virus, and fungi (Wahab et al. 2003). Among them, viral diseases (e.g. white spot syndrome disease (WSSD) and monodon baculovirus (MBV) disease) are severe, where >20 viruses have been reported to infect penaeid shrimp farms globally (Lightner 1996; Lightnert & Redman 1998; Flegel 2006). Next to the WSSD, loose-shell syndrome disease (LSSD) is nearly as serious as WSSD in penaeid shrimps, causing mass mortality. LSSD infection occurs in adults, but juveniles are infected more frequently. LSSD was first reported in India in 1998, and the incidence of this disease has increased every year (Alavandi et al. 2007, 2008). Clinical symptoms include a loose shell, soft muscle, and a condensed melanized hepatopancreas, which result in the reduction of daily growth rate, body weight loss, and poor survival of shrimps (Gopalakrishnan & Parida 2005; Loka et al. 2012). Other symptoms include loss of appetite, weak response to stimuli, and erratic swimming (Loka et al. 2012). The occurrence of LSSD in a shrimp farm is believed to be associated with such factors as nutritionally imbalanced feed, different chemical pesticides, deterioration of pond soil and water quality, and poor management practices (Baticados et al. 1986). The onset of LSSD in shrimp farms can be easily detected by changes in the exoskeleton as well as in the internal organs. Further, LSSD in shrimps can be caused by fungi, bacteria, and protozoa, creating an environment for the suspended particles to settle on the exoskeleton and appendage, making them vulnerable (Gopalakrishnan & Parida 2005).

Coastal aquaculture in Bangladesh is mostly extensive, where tiger shrimp *Peneaus monodon* is one of the primary species because of its large size and fast growth compared to other penaeid shrimps (Flegel 2006). The coastal shrimp industry in Bangladesh employs \(\sim 1.2\) million people and contributes

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~3.74% of the total GDP to the national economy (Paul & Vogl 2013). Recently, LSSD has become a severe problem in grow-out ponds in Bangladesh. Even though the LSSD of tiger shrimp is considered to be an emerging problem in India, very few studies have been conducted in Bangladesh. The causative agents of LSSD in brackish water ponds in Bangladesh are still unknown. With this in mind, the current research was carried out (i) to observe some clinical signs of LSSD to be confirmed about its incidence and (ii) to identify the probable causative agents for LSSD in brackish water ponds. Additionally, ecological parameters of LSSD-affected and non-affected shrimp ponds are also monitored.

Materials and methods

Study area and measurement of ecological parameters

Sampling was conducted randomly from five loose-shell-affected and three non-affected ponds (0.5–0.8 ha each) during July, September, and December 2013 near Bakkhali River, which is located in the southeastern part of Bangladesh between latitudes 21°27′N and 21°28′N and longitudes 91°57′E and 91°58′E (Figure 1). Ecological parameters such as salinity (g/kg), water temperature (°C), water pH, soil pH, dissolved oxygen (DO), and ammonia (NH₃-N) were measured by hand refractometer, digital thermometer, pen pH meter, soil tester, DO test kit (Model No. HI 98302, HANNA instruments products, Singapore), and ammonia test kit (Model No. HI 3812, HANNA instruments products, Singapore), respectively.

Shrimp samples and qualitative investigation

Weak and lethargic shrimp P. monodon weighing 20–30 g, aggregating at the edges of the ponds and showing progressive signs of LSSD, that is, crumpled exoskeleton and flexible abdomen, were collected. A total of 75 moribund (LSSD-affected) and 180 non-infected specimens (60 specimens from each pond) were collected and live-transported to the laboratory in oxygen-packed polythene bags with styrofoam cork sheet box. During the transportation (2 h to reach the laboratory), the water temperature was maintained at 26 ± 2°C by using an ice bag. The LSSD-affected and non-affected shrimps were dissected immediately in the laboratory. The gut, gill, and abdomen tissues were separated and Zoothamnium spp. and other epi-commensals attached on the shell surface and gill regions were noted. Identification of Zoothamnium spp. and isopods was done according to Hu and Song (2001) and Jones (1976).

Experimental design

Haemolymph samples (0.1 ml) were drawn from the ventral sinus of each shrimp with the help of a sterile syringe and plated on lukewarm trypticase soy agar (TSA) with 2% NaCl and thiosulphate citrate bile salts sucrose (TCBS) agar (Oxoid, Hampshire, UK) for total bacterial and total Vibrio counts, respectively, and incubated at 27°C for 48 h as suggested by Lightner (1996). The colonies of Vibrio spp. were enumerated with the help of a colony counter and expressed in colony-forming units (CFU) per milliliter of haemolymph. The dominant yellow and green bacterial colonies were picked and isolated by frequently streaking on TSA medium (Oxoid, Hampshire, UK), followed by storing the pure cultures in nutrient agar slants in a refrigerator at 4°C.

The bacterial examination was performed following the methods of Alsina and Blanch (1994a, 1994b) and Holt et al. (1994). In addition, API 20E and API 20NE (BioMérieux, France) test strips were used for further confirmation. The sensitivity of the isolated bacteria to 14 commercially available antibiotics was determined according to Kirby–Bauer disk diffusion method (Bauer et al. 1996). The bacteria were inoculated on Muller Hinton Agar (with 2% NaCl) and commercially available antibiotic Sensi-Discs were placed on the agar media. After 24 h growth, the inhibition zone around the discs was measured with a ruler. Sensitivity was categorized into three levels, that is, resistant, intermediate, and sensitive, according to Bauer et al. (1996).

For the challenge test, gill, cephalothorax, and muscle were collected from the LSSD-infected shrimps and homogenized by a glass homogenizer in sterilized phosphate-buffered saline (PBS) (1 g of tissue material in 1 ml PBS) and centrifuged at 2200 rpm for 10 min following the methods of Takahashi et al. (1994) and Mayavu et al. (2003). The supernatant was filtered through a 0.25-μm membrane filter and diluted in PBS (at a ratio of 1:10). In the first set of experiments, LSSD-affected shrimp extract was injected intramuscularly at a dose of 100, 50, and 10 μL into 3 × 30 non-affected shrimps (weighting ~15–20 g). For the control group, 30 non-affected individuals were used. In the second set of experiments (immersion method), LSSD-affected shrimp extract (1 × 10³ CFU g⁻¹) was dissolved in sterile seawater (20 g kg⁻¹) in the experimental glass tanks (10 L). Each experimental tank contained 10 individuals and 3 tanks were used for each treatment. For control, sterile PBS was injected into 10 individuals. Mortality was recorded on the 12th day of the experimental period for the first experiment and 13th day of the experimental period for the second experiment. During the experimental period, 10% of water was exchanged per day. Continuous aeration and commercial formulated feeds were given to shrimps in both control and experimental groups.

Test for MBV

Hepatopancreas from the LSSD-affected and non-affected shrimps were dissected and placed on oil-free glass slides. The samples were stained with 0.05% aqueous malachite green and squashed mildly with a cover slip and examined under a light microscope to detect representative viral occlusion bodies (Lightner 1996).

Test for WSSV

WSSV test was performed by a commercial insulated isothermal PCR (ii PCR), IQ Plus™, and WSSV kit with POCKIT System (GeneReach Biotechnology Corporations, Taiwan). DNA extraction and other procedures were done according to the instruction manual of the IQ Plus system.
Statistical analyses

All the ecological and production parameters data were analysed using STATISTICA v 12 (Statsoft Inc., Tulsa, OK, USA). Residuals were tested for normality (Shapiro–Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). Student t-test was performed to determine the differences in ecological parameters (i.e. temperature, water and soil pH, DO, and NH₃) and growth parameters (i.e. total biomass, mean daily growth rate, final body weight, feed conversion ratio (FCR), and mean % of survivability) between normal and LSSD-affected ponds. Statistical significance was tested at the 95% confidence level.

Results

Ecological parameters

There were no significant deviations for water temperature and salinity between normal and LSSD-affected ponds (Figure 2(A,B)). The mean value of water and soil pH (Figure 2(C)), DO, and NH₃-N (Figure 2(D)) showed different trends during the experimental period, but there were no significant differences between normal and LSSD-affected ponds.

Production analysis

The total shrimp production was higher in normal ponds (185 kg/ha) than in the LSSD-affected ponds (126–146 kg/ha), and survival of shrimp during harvest was significantly different (p < .01) between normal and LSSD-affected ponds (Table 1). Likewise, the mean body weight of the individuals at harvesting was lower in LSSD-affected shrimp ponds (25.9 g) than in the normal ponds (30.6 g), while the average FCR in LSSD-affected ponds (1.9) was poorer than that in the normal ponds.

Qualitative investigation

In five LSSD-affected shrimp ponds, more than 50% of the shrimps had empty guts, remarkably soft muscle, and loose and swelling exoskeleton. This was further ascertained by the
deficiency of faecal filament in the lift net. The intestine of infected individuals was opaque and milky white in colour. Mostly, juveniles (weighted 20–35 g) which were infected by LSSD remained lethargic and did not moult throughout the culture period. Some barnacle species fouled the carapace of ∼35% of infected individuals, and the exoskeleton and gills of few individuals were infested with *Zoothamnium* spp. (5%) and isopods (15%). Additionally, bio-foulers attached to the appendages of the infected individuals appeared as brown and black. However, most of the infected individuals were found to have a white-brown or brown-black thin carapace with a space between the muscle and the exoskeleton.

**Microbial study**

The prevalence of infection with WSSV and MBV was found to be lower than that with the *Vibrio* community. During the investigation, 8% LSSD-affected individuals were WSSV positive, 5% were MBV positive, and 4% had both infections, while 3% of LSSD-affected shrimps were infected by the association of *Vibrio* sp. and multiple viruses (WSSV and MBV) (Table 1). The present study found five *Vibrio* species, viz., *V. alginolyticus* (28%), *V. harveyi* (22%), *V. anguillarum* (15%), *V. vulnificus* (11%), and *V. splendidus* (12%), mostly from LSSD-affected shrimps (Figure 3). Among the 82 *Vibrio* isolates, the present study revealed *V. alginolyticus* as the most dominant species, followed by *V. harveyi* and others from LSSD-affected and non-affected shrimps. *V. alginolyticus* and *V. splendidus* were found in all diseased shrimps (Figure 3). The occurrence of *V. alginolyticus* and *V. splendidus* was rare in non-affected shrimp farms.

In the first set of experiments, where individuals were injected with 1 mL of LSSD-affected shrimp extract, mortality started 2 days after the injection and all individuals died within 7 days. For 0.5 and 0.1 mL extract injected groups, mortality of the individuals started between 4 and 6 days after the injection and all the individuals were dead within 9–12 days (Figure 4(A)). In the second set of experiments, individuals exposed to LSSD-affected shrimp extract showed 20% mortality initially at day 5 and the final total mortality was 100% at 13 days (Figure 4(B)). The first indicator of LSSD in the affected

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**Table 1.** Growth parameters and the rate of viral infection of tiger prawn in normal and LSSD-affected ponds.

| Description of data                     | Normal pond | LSSD-affected pond |
|-----------------------------------------|-------------|---------------------|
| Source of water                         | Tidal       | Tidal              |
| Rate of water exchange (%)              | 15          | 15                 |
| Total Biomass (kg/hac⁻¹)                | 185ᵃ        | 146ᵇ               |
| Culture Period                          | 140         | 140                |
| Mean daily growth rate (g)              | 0.22        | 0.17               |
| Final average body weight (g)           | 30.6 (±2.3)ᵃ| 25.9 (±2.5)ᵇ      |
| FCR                                     | 1.6 (±0.1)ᵇ| 1.9 (±0.1)ᵇ        |
| Survival at harvest (%)                 | 70.8ᵃ       | 50.7ᵇ              |
| WSSV infection (%)                      | 2           | 8                  |
| MBV infection (%)                       | 3           | 5                  |
| Double viral (WSSV and MBV) (%)         | Nil         | 4                  |
| Multiple viral (WSSV, MBV, and Vibrio sp) | Nil       | 3                  |

Note: Different letters within rows indicate significant differences.
individuals was the cessation of feed consumption, where more than 50% of the individuals had an empty gut. The intestine of LSSD-infected individuals turned into opaque milky white colour and remarkably had tender and swollen exoskeleton. Most of the affected shrimps were white-brown in colour and had a thin carapace, with a space between the muscle and the exoskeleton. The microbiological analysis showed that the total number of *Vibrio* increased in the haemolymph from $2\times 10^3$ to $2\times 10^6$ CFU g$^{-1}$ for the uninfected individuals to $6\times 25 \times 10^6$ CFU g$^{-1}$ for the LSSD-infected individuals. The morphological and biochemical characteristics of the five *Vibrio* species isolated from the LSSD-affected shrimp are presented in Table 2, where *V. alginolyticus* was the dominant species compared to other *Vibrio* species. The drug sensitivity experiment demonstrated that most of the isolated bacteria were sensitive to chloramphenicol, norfloxacin, and ciprofloxacin, but susceptible to penicillin, ampicillin, and amoxicillin (Table 3).

**Discussion**

In the present study, the occurrence of LSSD in the brackish water shrimp ponds was suspected by a number of indicators including cessation of feed consumption, empty gut, milky white intestine, fuzzy mat due to protozoa, brownish gill, and papery-carapace and infestation with fouling organisms (barnacles, isopods, *Zoothamnium* spp., etc.). The symptoms of LSSD-affected individuals were similar to the previous reports of LSSD in tiger shrimp from India (Lightner & Redman 1998; Gopalakrishnan & Parida 2005; Alavandi et al. 2008; Kalaimani et al. 2013). The predominant *Vibrio* species found in the cultured shrimps have developed as the active pathogen when the immune system of the shrimps is suppressed (Brock & Lightner 1990; Kumar et al. 2014).

The optimum temperature for the grow-out ponds ranges from 25°C to 28°C. The greater range of temperature (24.5–33°C) recorded in the present study from LSSD-affected ponds might have contributed to the mortality of the shrimps (Boyd & Fast 1992). The range of salinity from the LSSD-affected ponds (22–34 g/kg) was higher than the optimum range of salinities (15–25 g/kg) for shrimp grow-out ponds (Chen 1985; Boyd 1989). The levels of DO determined in both normal (3.0–5.2 mg/L) and LSSD-affected ponds (2.3–4.2 mg/L) were within the range recommended by Liao and Murai (1986), but with some values lower than that recommended of 4.0 mg/L. The level of DO should be routinely maintained at >4 mg/L in order to avoid metabolic stress of shrimps in grow-out ponds (Hall & Van Hamm 1998). The recommended water pH is 7.5–8.5, which is close to that observed in normal and LSSD-affected ponds (Law 1988). The tiger shrimp can tolerate a pH of 6.0, but lower levels might cause mortality. In the present study, the mean concentration of ammonia from the LSSD-affected ponds varied from 0.31 to 1.35 mg/L, which exceeded the recommended value of 0.15 mg/L (Law 1988). The mortality rate in the LSSD-affected ponds was probably influenced by this high concentration of ammonia (Emerson et al. 1975).

The mean daily growth rate of tiger shrimp in LSSD-affected ponds was lower than that in the normal ponds, but not significantly different. The estimated mean daily growth for normal (0.22 g) and LSSD-affected ponds (0.17 g) was higher than the previous report (0.17–0.18 g) obtained from the Indian coast (Santiago 1977; Gopalakrishnan & Parida 2005). The average survival rate in the LSSD-affected ponds was significantly lower (50.67%) than that in the normal ponds ($p < .01$). Previous study showed that tiger shrimp possessed cannibalistic behaviour (Gopalakrishnan & Parida 2005) and due to this behaviour, infectious diseases can be transmitted from one individual to another very rapidly. The total production of LSSD-affected ponds (126–146 kg/ha) was significantly lower ($p < .05$) than that of the normal ponds, which is clearly an indication of the effects of the incidence of LSSD in our experimental ponds (Gopalakrishnan & Parida 2005; Alavandi et al. 2008). Even though the causal agent of LSSD is not yet clearly recognized, the occurrence of many harmful bacteria can be responsible for the incidence of LSSD in our brackish water shrimp ponds.

The presence of *Vibrio* species in shrimp grow-out ponds can be attributed to multiple etiological agents (Raja et al. 2012). Several studies reported that nutritionally imbalanced shrimp

**Figure 3.** Prevalence of *vibrio* species in LSSD-affected and non-affected shrimp ponds.

**Figure 4.** Mortality of individuals due to experimental infection by LSSD-affected shrimps extracts directly injected to experimental shrimps (A) and mixed in water (B).
feed, susceptibility to different chemical pesticides, deterioration of pond soil and water quality, and poor management practices can be responsible for LSSD in shrimps (Baticados et al. 1986). We recorded 100% mortality due to intramuscularly inoculated LSSD-affected shrimp extracts in the present study. The rapid mortality in the injected healthy individuals in our study may be due to the increasing concentration of pathogens in the filter. Drug sensitivity studies indicated that all the *Vibrio* spp. were susceptible to chloramphenicol, norfloxacin, and ciprofloxacin and insusceptible to penicillin, ampicillin, and amoxicillin. The findings of our drug sensitivity experiments correspond well to the outcome of sensitivity studies observed by several researchers (Corliss et al. 1977; Mohney et al. 1992; Ruangpan & Kitao 1992; Charantchakool et al. 1995). Furthermore, *V. harveyi* exhibited high resistance to chloramphenicol and was highly sensitive to ciprofloxacin, norfloxacin, and oxytetracycline (Karunasagar et al. 1994).

Table 2. Morphological and biochemical characteristics of *vibrio* spp. isolated from LSSD-affected shrimps.

| Tests                      | *V. alginolyticus* (*N* = 24) | *V. harveyi* (*N* = 21) | *V. anguillarum* (*N* = 15) | *V. vulnificus* (*N* = 12) | *V. splendidus* (*N* = 10) |
|----------------------------|-------------------------------|--------------------------|-----------------------------|-----------------------------|-----------------------------|
| Gram staining              | –                             | –                        | –                           | –                           | –                           |
| Growth on TCBS             | Y                             | Y                        | Y                           | Y                           | Y                           |
| Motility                   | +                             | +                        | +                           | +                           | +                           |
| Oxidase reduction          | +                             | +                        | +                           | +                           | –                           |
| Catalase reduction         | +                             | +                        | +                           | +                           | –                           |
| O/F test                   | F                             | F                        | F                           | F                           | F                           |
| Arginine dihydrolase       | –                             | –                        | +                           | –                           | +                           |
| Lysine decarboxylase       | +                             | +                        | –                           | –                           | –                           |
| Ornithine decarboxylase    | +                             | +                        | –                           | –                           | –                           |
| Methy1 red test            | +                             | +                        | –                           | –                           | –                           |
| Voges proskauer            | +                             | +                        | +                           | –                           | –                           |
| Indole production          | +                             | +                        | +                           | +                           | +                           |
| Nitrate reduction          | +                             | +                        | +                           | +                           | +                           |
| Citrate utilization        | +                             | +                        | +                           | +                           | +                           |
| Gelatinase                 | +                             | +                        | +                           | +                           | +                           |
| Esculin hydrolysis         | +                             | +                        | –                           | +                           | +                           |
| Sensitivity to O/129 (10 µg)| +                             | +                        | –                           | +                           | +                           |
| O/129 (150 µg)             | +                             | +                        | +                           | +                           | +                           |
| Growth at 4°C              | –                             | –                        | –                           | –                           | –                           |
| Growth at 42°C             | –                             | –                        | –                           | –                           | –                           |
| Growth with NaCl (%)       | –                             | –                        | –                           | –                           | –                           |
| 0                          | –                             | –                        | –                           | –                           | –                           |
| 0.5                        | +                             | +                        | –                           | +                           | +                           |
| 1                          | +                             | +                        | +                           | +                           | +                           |
| 3                          | +                             | +                        | +                           | +                           | +                           |
| 6                          | +                             | +                        | +                           | +                           | +                           |
| 8                          | +                             | +                        | –                           | +                           | +                           |
| 10                         | –                             | –                        | –                           | –                           | –                           |
| Acid production from       |                               |                          |                             |                             |                             |
| Arabinose                  | –                             | –                        | –                           | –                           | –                           |
| Cellobiose                 | –                             | +                        | –                           | –                           | –                           |
| Galactose                  | +                             | +                        | +                           | –                           | –                           |
| Glucose                    | +                             | +                        | +                           | +                           | +                           |
| Inositol                   | –                             | –                        | –                           | –                           | –                           |
| Lactose                    | –                             | –                        | +                           | +                           | +                           |
| Manitol                    | +                             | +                        | +                           | –                           | +                           |
| Sorbitol                   | +                             | +                        | +                           | –                           | +                           |
| Sucrose                    | +                             | +                        | +                           | +                           | +                           |
| Urease                     | +                             | –                        | –                           | +                           | +                           |
| H₂S production             | –                             | +                        | –                           | –                           | –                           |

Note: + = 90–100% strains positive; – = 0–10% strains positive; F = fermentative; y = yellow.

Table 3. Sensitivity of *vibrio* spp. isolated from LSSD-affected shrimps to several antibiotics.

| Antibiotics               | *V. alginolyticus* (*N* = 15) | *V. anguillarum* (*N* = 10) | *V. harveyi* (*N* = 15) | *V. vulnificus* (*N* = 10) |
|---------------------------|-------------------------------|-----------------------------|--------------------------|-----------------------------|
| Ampicillin (10 µg)        | R                             | R                           | R                        | R                           |
| Amoxicillin (30 µg)       | R                             | R                           | R                        | R                           |
| Chloramphenicol (30 µg)   | S                             | S                           | R                        | S                           |
| Erythromycin (15 µg)      | I                             | R                           | R                        | I                           |
| Ciprofloxacin (5 µg)      | S                             | S                           | S                        | S                           |
| Furazolidon (20 µg)       | R                             | R                           | I                        | I                           |
| Nitrofurazolidon (30 µg)  | R                             | I                           | R                        | R                           |
| Norfloxacin (10 µg)       | S                             | S                           | S                        | S                           |
| Metronidazol (20 µg)      | R                             | R                           | R                        | R                           |
| Oxytetracyclin (20 µg)    | I                             | I                           | S                        | I                           |
| Penicillin G (10U)        | R                             | R                           | R                        | R                           |
| Co-trimoxazole (10 µg)    | R                             | S                           | R                        | R                           |
| Streptomycin (10 µg)      | R                             | R                           | R                        | R                           |
| Tetracycline (20 µg)      | I                             | S                           | R                        | R                           |

Note: S = Sensitive (18–25 mm), R = Resistant (6–12 mm), I = Intermediate (13–17 mm).
Conclusions

The present investigation clearly revealed the manifestation of LSSD in our grow-out shrimp ponds in Bangladesh. Although the presence of WSSV and MBV was examined in our LSSD-affected ponds, we did not confirm the influence of WSSV and MBV on the incidence of LSSD in our shrimp ponds. The occurrence of LSSD can be due to the bacterial infection as we observed several bacterial species in LSSD-affected shrimps. However, our study suggested that the prevalence of LSSD in tiger prawn can also be associated with poor soil and water quality, and the deficient management of the ponds.

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Disclosure statement

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