Yumbah Nyamat – Refining the development of a large scale production system for the sustainable production of abalone

Effects of diluted chemical disinfectants on abalone followed by the disinfectants neutralisation in seawater

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Introduction

The present trial is part of a larger project which aims to identify a chemical disinfectant that inactivates *Haliotid herpesvirus 1* (HaHV), is not harmful to abalone and can be used in a recirculating aquaculture system. This chemical must have the potential to be approved by the Australian Pesticides and Veterinary Medicines Authority (APVMA) to allow its use on abalone. A high treatment efficacy against HaHV needs to be achieved while abalone health, farm staff health and safety and environmental safety are not compromised.

Chemical disinfectants against Haliotid and related herpesviruses

Chemical disinfectants such as calcium hypochlorite (providing 65–70 % available chlorine), the non-ionic surfactant *Impress* and the iodophor *Buffodine* were tested against HaHV under laboratory conditions and effectively inactivated the virus (Corbeil et al., 2012). Corbeil et al. (2012) found that 2 ppm of residual chlorine inactivated HaHV during a 15-minute exposure in seawater at 16 °C. Kasai et al. (2005) recommend 3 ppm of residual chlorine for a 20-minute exposure to inactivate *Cyprinid herpesvirus 3* (CyHV-3). The effectiveness of chlorine is significantly reduced in the presence of organic matter in the water (Department of Agriculture Fisheries and Forestry, 2008). For example, 50 ppm of available chlorine inactivated *Ostreid herpesvirus 1* (OsHV-1) during a 15-minute exposure in relatively clean seawater; but, in other treatment, when organic matter in the form of 10% foetal bovine serum was added and mixed in the seawater, 50 ppm of available chlorine was not effective against OsHV-1 (Hick et al., 2016). 'Available' refers to the concentration of active component in the solution at the start of a disinfection process; and, 'residual' refers to the level of active component remaining in the solution during and at completion of a disinfection process (Department of Agriculture Fisheries and Forestry, 2008).

Buffodine (containing 1–3 % iodine) at concentration of 50 ppm inactivated HaHV placed in an experimental tube (2 ml volume) after 10 minutes incubation (Corbeil et al., 2012). *Betadine* (containing 10 % povidone iodine equivalent to 1 % iodine) at concentration of 0.1 % available iodine inactivated OsHV-1 in oyster tissue during a 5-minute exposure (Hick et al., 2016). Other chemical disinfectants such as chlorine dioxide and hydrogen peroxide are also used to inactivate virus (Smail et al., 2004; Department of Agriculture Fisheries and Forestry, 2008; Zychem Technologies, 2011; Bowker et al., 2014); although, to our knowledge, they have not been tested against HaHV and OsHV-1.

Chemical disinfectants and recirculating aquaculture systems

Some chemical disinfectants and medicine used to treat water and fish can be harmful to nitrifying bacteria in the biofilter, negatively affecting its function and potentially causing substantial ammonia and/or nitrite accumulation (Helfrich and Libey, 1991; Noble and

Summerfelt, 1996; Pedersen et al., 2009). If a chemical concentration can be harmful to the biofilter, the chemical should be used at a lower concentration for a more prolonged treatment period or the biofilter should be by-passed during treatment and the tanks flushed with fresh water prior to re-establishing flow to the biofilter (Noble and Summerfelt, 1996). However, it is possible that an untreated biofilter can become a reservoir for the disease organism (Noble and Summerfelt, 1996). It is known that high concentrations of hydrogen peroxide (e.g. 100 ppm) can have detrimental effects on biofilter performance, calling for treatments with low dose applications (Noble and Summerfelt, 1996; Pedersen and Pedersen, 2012).

Neutralisation of residual chemicals and recirculating aquaculture system

In a recirculating aquaculture system, after a treatment with disinfectant, it is important that residual chemical is neutralised and/or removed from the water. Sodium thiosulphate is a non-hazardous chemical that neutralises chlorine, iodine and chlorine dioxide (Ferweda, 2004; World Organization for Animal Health, 2009). To neutralise chlorine, the amount of sodium thiosulphate should be 2.85 times the amount of chlorine; and, to neutralise iodine, the amount of sodium thiosulphate should be 0.78 times the amount of iodine (World Organization for Animal Health, 2009). Optimal neutralisation of chlorinated water is achieved by passage through activated carbon which removes excess chlorine and chloramines (World Organization for Animal Health, 2009). Activated carbon also removes benzalkonium chloride from treated water (Tanada et al., 1991).

Commented [C1]: This chemical also needs to be approved by APVMA

Commented [C2]: Has to be a non-hazardous chemical

Activated carbon treated with concentrated sulphuric acid improves the removal of hydrogen peroxide in aqueous solutions (Ribeiro et al., 2013). However, safety must be considered when using activated carbon with high concentrations of hydrogen peroxide (Jones, 2007). The oxygen released during the decomposition of hydrogen peroxide could react with activated carbon and cause an explosion (Jones, 2007).

Trial

The present trial was divided into two parts as described in this report.

Part 1: Bath treatment and monitoring period

Objective

The objective of Part 1 was to assess the effects of diluted calcium hypochlorite, the iodophors *Buffodine* and *Ovadine*, the non-ionic surfactant *EnviroClean*, the chlorine dioxide *Zydox* and hydrogen peroxide 50 % on the abalone behaviour, health and survival.

Material and methods

Chemical disinfectants

The chemical disinfectants selected for this trial were the non-ionic surfactant *EnviroClean* (enviroCARE EARTH), calcium hypochlorite (Sigma-Aldrich), the iodophors *Buffodine* (Evans Vanodine International) and *Ovadine* (Future Fisheries Veterinary Services Pty Ltd), the chlorine dioxide *Zydox* (Zychem Technologies Pty Ltd) and hydrogen peroxide 50 % (Ace Chemical Company).

EnviroClean contains a maximum of 5 % benzalkonium chloride. Calcium hypochlorite provides a minimum of 65 % available chlorine. *Buffodine* contains a maximum of 3 % iodine and 10–15 % alcohol ethoxylate (a non-ionic surfactant). *Ovadine* contains 10 % povidone iodine complex, which provides 1 % available iodine.

Experimental system

Laboratory was equipped with 14 x 10-L tubs (12 for bath treatments and 2 for control groups) connected to a flow-through seawater system running at ambient seawater temperature. Seawater was filtered to 1 µm before reaching the tubs. Each tub had one airstone for aeration.

Bath treatments and control groups

Twelve bath treatments were undertaken between 19th and 21st June 2018. Two control groups were held during the same period. A total of 112 greenlip abalone *Haliotis laevigata* were used, being 8 abalone for each bath treatment and control group. Abalone were on average 63.7 ± 3.0 mm (mean ± standard deviation (SD)) in shell length and 39.2 ± 5.7 g in weight. Changes in abalone behaviour, visible side effects and mortality during the bath treatments were recorded. Abalone in the control groups were left undisturbed. All abalone were unfed between 19th and 21st June 2018.

The chemical disinfectants were added into the tubs to obtain the final concentrations specified in Table 1. The duration of each bath treatment was 60 minutes. During this period, each tub had 8 litres of treated seawater (seawater plus chemical disinfectant), the seawater flow was off and the aeration was on. Seawater temperature, pH and oxygen saturation were measured and recorded prior, during and after the bath treatments. Seawater temperature and oxygen saturation were on average 12.5 ± 0.5 °C and 97.5 ± 1.4 %, respectively.

Table 1

Summary of the bath treatments (*n*= number of abalone; information in parentheses means mean shell length ± SD in mm and mean weight ± SD in g).

Bath treatment / Chemical	Tub number	*n*	Concentration
Non-ionic surfactant *EnviroClean*	1	8 (63.9±4.1mm; 40.9±6.2g)	1%
Calcium hypochlorite	2	8 (65.3±3.8mm; 40.4±7.5g)	15 ppm
Calcium hypochlorite	7	8 (64.8±2.9mm; 38.3±4.0g)	25 ppm
Iodophor *Buffodine*	3	8 (64.0±3.5mm; 41.6±7.6g)	50 ppm
Iodophor *Buffodine*	8	8 (62.8±2.2mm; 37.8±5.5g)	150 ppm
Iodophor *Buffodine*	13	8 (61.4±2.7mm; 34.6±6.1g)	250 ppm
Iodophor *Ovadine*	6	8 (64.0±2.2mm; 39.9±4.4g)	50 ppm
Iodophor *Ovadine*	9	8 (63.6±1.8mm; 37.5±3.3g)	150 ppm
Chlorine dioxide *Zydox*	4	8 (65.6±3.2mm; 43.4±6.6g)	25 ppm
Chlorine dioxide *Zydox*	11	8 (63.1±3.0mm; 36.6±4.7g)	50 ppm
Hydrogen peroxide 50%	5	8 (64.4±2.8mm; 42.5±6.1g)	25 ppm
Hydrogen peroxide 50%	12	8 (63.4±3.2mm; 38.8±4.2g)	50 ppm

The levels of residual chemicals were measured and recorded every 10 minutes during the 60-minutes bath treatments. During the bath treatment with the non-ionic surfactant *EnviroClean*, the level of residual benzalkonium chloride was measured using *Quantofix QUAT* test strips with range of 0–1000 ppm (gradation is 0, 10, 25, 50, 100, 250, 500, 1000). During the bath treatments with calcium hypochlorite, the level of residual free chlorine was measured using *Hach Aquacheck* test strips with range of 0–10 ppm (gradation is 0, 0.5, 1, 2, 4, 10) and *WaterWorks* test strips with range of 0–750 ppm (gradation is 0, 25, 50, 100, 200, 400, 500, 750). During the bath treatments with the iodophors *Buffodine* and *Ovadine*, the levels of residual iodine were measured using *WaterWorks* test strips with range of 0–300 ppm (gradation is 0, 5, 10, 15, 20, 30, 40, 50, 75, 100, 150, 200, 250, 300). Visible changes in colour of the abalone foot due to the iodine were recorded. During the bath treatments with the chlorine dioxide *Zydox*, the level of residual chlorine dioxide was measured using *Insta-Test Analytic* test strips with range of 0–500 ppm (gradation is 0, 10, 25, 50, 100, 250, 500). During the bath treatments with hydrogen peroxide 50 %, the level of residual hydrogen peroxide was measured using *WaterWorks* test strips with range of 0.5–100 ppm (gradation is 0.5, 2, 5, 10, 25, 50, 100).

After completion of each bath treatment, the treated seawater (seawater plus chemical disinfectant) was poured into a plastic bucket for the neutralisation treatment (described in Part 2 of this report). The empty tub containing abalone was immediately filled with fresh untreated seawater and the seawater flow was turned on.

Monitoring period

After completion of the bath treatments, all abalone (including the ones in the control groups) were left in the tubs and monitored for 15 days, between 22nd June and 6th July 2018. Changes in abalone behaviour, visible side effects, numbers of moribund abalone and mortality during this period were recorded. Abalone were fed during the monitoring period. Feeding and cleaning procedures and frequencies were carried out according to normal farm practices. Seawater temperature and oxygen saturation were measured and recorded, being on average 13.0 ± 0.5 °C and 96.7 ± 2.3 %, respectively. At the end of the monitoring period, from each bath treatment, some abalone were fixed in formalin and some abalone were frozen. If necessary, fixed abalone will be submitted for histological analysis and frozen abalone for chemical residue testing. Abalone from the control groups were moved back to the farm.

Results

Bath treatments and control groups

Bath treatment with the non-ionic surfactant EnviroClean at concentration of 1 %

Changes in behaviour were observed within 10 minutes of treatment. The abalone extended their adductor muscles leading to elevation of the shell, and they swivelled the shell around the muscle axis (Video 1). After 20 minutes of treatment, this behaviour was significantly reduced to small and slow movements of the foot of some abalone (Video 2), and all abalone lost their adhesion to the tub surface. Between 30 minutes and the end of treatment (60 minutes), the abalone did not move and were detached from the tub surface. After the end of treatment, the treated seawater was changed to fresh untreated seawater, and two abalone were trying to reattach to the tub surface. No mortality was recorded.

The initial level of residual benzalkonium chloride was around 250 ppm and it did not change throughout the treatment. Seawater temperature and oxygen saturation during the treatment were on average 12.8 ± 0.2 °C and 97.5 ± 0.7 %, respectively. *EnviroClean* at concentration of 1 % dropped the pH from 8 to 7.

Bath treatment with calcium hypochlorite at concentration of 15 ppm

Changes in behaviour were observed within 10 minutes of treatment. The abalone extended their adductor muscles leading to elevation of the shell, and they swivelled the shell around the muscle axis. However, the extent of these movements in comparison with the movements observed in the bath treatment with the non-ionic surfactant *EnviroClean* was significantly less strong. After 20 minutes until the end of treatment (60 minutes), small and slow movements of the foot of some abalone were observed, and all abalone were

detached from the tub surface. After the end of treatment, the treated seawater was changed to fresh untreated seawater, and the abalone were trying to reattach to the tub surface. No mortality was recorded.

The initial level of residual free chlorine was around 10 ppm and it did not change throughout the treatment. Seawater temperature and oxygen saturation during the treatment were on average 12.7 ± 0.2 °C and 96.4 ± 0.5 %, respectively. Calcium hypochlorite at concentration of 15 ppm dropped the pH from 8 to 6.

Bath treatment with calcium hypochlorite at concentration of 25 ppm

Changes in abalone behaviour were very similar to the changes described above for the bath treatment with calcium hypochlorite at concentration of 15 ppm. All abalone were detached from the tub surface after 20 minutes of treatment. At the end of treatment (60 minutes), all abalone were alive and two abalone were attached to the tub surface.

The initial level of residual free chlorine was between 10–25 ppm (probably around 17 ppm) and it did not change throughout the treatment. Seawater temperature and oxygen saturation during the treatment were on average 12.3 ± 0.1 °C and 98.7 ± 0.7 %, respectively. Calcium hypochlorite at concentration of 25 ppm dropped the pH from 8 to 6.

Bath treatment with the iodophor Buffodine at concentration of 50 ppm

No changes in abalone behaviour, such as movement of the foot and elevation of the shell, were observed. They did not lose their adhesion to the tub surface from the beginning to the end of the treatment (60 minutes). No mortality was recorded. The initial level of residual iodine was between 0.1–5 ppm (probably around 1 ppm) and it did not change until the end of the treatment. Seawater temperature and oxygen saturation during the treatment were on average 11.6 ± 0.1 °C and 96.6 ± 0.2 %, respectively. *Buffodine* at concentration of 50 ppm did not change the pH (pH = 8). Bubbles were observed on the seawater surface due to the non-ionic surfactant contained in *Buffodine*. Seawater and the abalone foot were not tanned by the iodine.

Bath treatment with the iodophor Buffodine at concentration of 150 ppm

No changes in abalone behaviour were observed, like in the bath treatment with *Buffodine* at concentration of 50 ppm above. The abalone did not move from the beginning to the end of the treatment (60 minutes), however, four abalone were detached from the tub surface at the end of the treatment. After changing the treated seawater to fresh untreated seawater, these abalone started trying to reattach to the tub surface. No mortality was recorded.

The initial level of residual iodine was between 0.1–5 ppm (probably around 3 ppm) and it did not change throughout the treatment. Seawater temperature and oxygen saturation during the treatment were on average 12.4 ± 0.1 °C and 98.2 ± 1.3 %, respectively. *Buffodine* at concentration of 150 ppm did not change the pH (pH = 8). Bubbles were observed on the seawater surface due to the non-ionic surfactant contained in *Buffodine*. Seawater and the abalone foot were not tanned by the iodine.

Bath treatment with the iodophor Buffodine at concentration of 250 ppm

Changes in behaviour, such as slow movements of the foot and elevation of the shell, were observed within 20 minutes of treatment (Video 3). The abalone started losing adhesion to the tub surface. After 30 minutes until the end of treatment (60 minutes), the abalone did not move. Five abalone were detached from the tub surface at the end of the treatment. After changing the treated seawater to fresh untreated seawater, these abalone started trying to reattach to the tub surface. No mortality was recorded.

The initial level of residual iodine was between 0.1–5 ppm (probably around 5 ppm) and it did not change throughout the treatment. Seawater temperature and oxygen saturation during the treatment were on average 13.0 ± 0.1 °C and 97.7 ± 0.6 %, respectively. *Buffodine* at concentration of 250 ppm increased the pH from 8 to 8.5. A large amount of bubbles was observed on the seawater surface due to the non-ionic surfactant contained in *Buffodine*. Seawater was slightly tanned by the iodine but the abalone foot was not.

Bath treatment with the iodophor Ovadine at concentration of 50 ppm

No changes in abalone behaviour, such as movement of the foot and elevation of the shell, were observed. They did not lose their adhesion to the tub surface from the beginning to the end of the treatment (60 minutes). No mortality was recorded. The initial level of residual iodine was between 0.1–5 ppm (probably around 0.5 ppm) and it did not change until the end of the treatment. Seawater temperature and oxygen saturation during the treatment were on average 12.9 ± 0.1 °C and 94.9 ± 1.4 %, respectively. *Ovadine* at concentration of 50 ppm did not change the pH (pH = 8). Seawater and the abalone foot were not tanned by the iodine.

Bath treatment with the iodophor Ovadine at concentration of 150 ppm

No changes in abalone behaviour, such as movement of the foot and elevation of the shell, were observed. They did not lose their adhesion to the tub surface from the beginning to the end of the treatment (60 minutes). No mortality was recorded. The initial level of residual iodine was between 0.1–5 ppm (probably around 1.5 ppm) and it did not change until the end of the treatment. Seawater temperature and oxygen saturation during the

treatment were on average 12.7 ± 0.1 °C and 97.3 ± 0.7 %, respectively. *Ovadine* at concentration of 150 ppm did not change the pH (pH = 8). Seawater and the abalone foot were not tanned by the iodine.

Bath treatment with the chlorine dioxide Zydox at concentration of 25 ppm

Changes in behaviour, such as slow movements of the foot and elevation of the shell, were observed within 20 minutes of treatment. Movements stopped after 30 minutes of treatment, and one abalone lost their adhesion to the tub surface after 40 minutes of treatment. At the end of the treatment (60 minutes), after changing the treated seawater to fresh untreated seawater, this abalone started trying to reattach to the tub surface. No mortality was recorded.

The initial level of residual chlorine dioxide was around 25 ppm and it did not change throughout the treatment. Seawater temperature and oxygen saturation during the treatment were on average 11.8 ± 0.1 °C and 96.7 ± 0.4 %, respectively. Chlorine dioxide *Zydox* at concentration of 25 ppm dropped the pH from 8 to 6.

Bath treatment with the chlorine dioxide Zydox at concentration of 50 ppm

Changes in behaviour were observed within 10 minutes of treatment. The abalone extended their adductor muscles leading to elevation of the shell, and they swivelled the shell around the muscle axis. However, the extent of these movements in comparison with the movements observed in the bath treatment with the non-ionic surfactant *EnviroClean* was significantly less strong. After 20 minutes of treatment, the movements stopped, some abalone lost their adhesion to the tub surface and some abalone kept their shell elevated until the end of the treatment (60 minutes). At the end of the treatment, all abalone were detached from the tub surface. No mortality was recorded.

The initial level of residual chlorine dioxide was around 50 ppm and it did not change throughout the treatment. Seawater temperature and oxygen saturation during the treatment were on average 12.5 ± 0.2 °C and 98.4 ± 0.4 %, respectively. Chlorine dioxide *Zydox* at concentration of 50 ppm dropped the pH from 8 to 6.

Bath treatment with hydrogen peroxide 50 % at concentration of 25 ppm

No changes in abalone behaviour, such as movement of the foot and elevation of the shell, were observed. They did not lose their adhesion to the tub surface from the beginning to the end of the treatment (60 minutes). No mortality was recorded. The initial level of residual hydrogen peroxide was around 25 ppm and it did not change until the end of the treatment. Seawater temperature and oxygen saturation during the treatment were on

average 12.9 ± 0.1 °C and 98.3 ± 0.2 %, respectively. Hydrogen peroxide 50% at concentration of 25 ppm did not change the pH (pH = 8).

Bath treatment with hydrogen peroxide 50 % at concentration of 50 ppm

No changes in abalone behaviour, such as movement of the foot and elevation of the shell, were observed. At the end of the treatment, three abalone were detached from the tub surface. No mortality was recorded. The initial level of residual hydrogen peroxide was around 50 ppm and it did not change until the end of the treatment. Seawater temperature and oxygen saturation during the treatment were on average 12.9 ± 0.1 °C and 99.4 ± 0.6 %, respectively. Hydrogen peroxide 50 % at concentration of 50 ppm increased the oxygen saturation slightly and did not change the pH (pH = 8).

Control groups 1 and 2

No changes in abalone behaviour and mortality were observed. Seawater temperature in the control groups 1 and 2 were on average 12.5 ± 0.5 °C and 12.8 ± 0.3 °C, respectively. Oxygen saturation in the control groups 1 and 2 were on average 97.2 ± 1.4 % and 98.5 ± 0.9 %. In both groups, pH was 8.

Monitoring period

Abalone from the bath treatment with the non-ionic surfactant EnviroClean at concentration of 1 %

All abalone were dead on the first day after the bath treatment.

Abalone from the bath treatment with calcium hypochlorite at concentration of 15 ppm

All abalone were alive and attached to the tub surface from the beginning to the end of the monitoring period.

Abalone from the bath treatment with calcium hypochlorite at concentration of 25 ppm

On the first five days after the bath treatment, one abalone was detached from the tub surface and two moribund abalone were recorded. These abalone recovered within the monitoring period. The rest of the abalone were alive and attached to the tub surface. No mortality was recorded.

Abalone from the bath treatment with the iodophor Buffodine at concentration of 50 ppm

One moribund abalone was recorded on the sixth day after the bath treatment. This abalone recovered within the monitoring period. The rest of the abalone were alive and attached to the tub surface.

Abalone from the bath treatment with the iodophor Buffodine at concentration of 150 ppm

All abalone were alive and attached to the tub surface from the beginning to the end of the monitoring period.

Abalone from the bath treatment with the iodophor Buffodine at concentration of 250 ppm

One abalone was detached from the tub surface on the first day after the bath treatment. After that, all abalone were attached to the tub surface until the end of the monitoring period. No mortality was recorded.

Abalone from the bath treatment with the iodophor Ovadine at concentration of 50 ppm

One moribund abalone was recorded during the monitoring period but it recovered. The rest of the abalone were alive and attached to the tub surface. No mortality was recorded.

Abalone from the bath treatment with the iodophor Ovadine at concentration of 150 ppm

Two abalone were detached from the tub surface on the first day after the bath treatment. After that, all abalone were attached to the tub surface until the end of the monitoring period. No mortality was recorded.

Abalone from the bath treatment with the chlorine dioxide Zydox at concentration of 25 ppm

Three moribund abalone were recorded during the monitoring period. Two of them recovered but the other one was still unhealthy at the end of the monitoring period. The rest of the abalone were alive and attached to the tub surface. No mortality was recorded.

Abalone from the bath treatment with the chlorine dioxide Zydox at concentration of 50 ppm

Five moribund abalone were recorded during the monitoring period. One of them died, two recovered and the other two were still unhealthy at the end of the monitoring period. One mortality occurred.

Abalone from the bath treatment with hydrogen peroxide 50 % at concentration of 25 ppm

Two moribund abalone were recorded during the monitoring period. One of them recovered but the other one was still unhealthy at the end of the monitoring period. The rest of the abalone were alive and attached to the tub surface. No mortality was recorded.

Abalone from the bath treatment with hydrogen peroxide 50 % at concentration of 50 ppm

Three abalone were detached from the tub surface on the first day after the bath treatment. After that, all abalone were attached to the tub surface until the end of the monitoring period. No mortality was recorded.

Abalone from the control group 1

All abalone were alive and attached to the tub surface from the beginning to the end of the monitoring period.

Abalone from the control group 2

All abalone were alive and attached to the tub surface from the beginning to the end of the monitoring period, apart from one moribund abalone.

Part 2: Neutralisation treatment

Objective

The objective of Part 2 was to assess the neutralisation process of each chemical disinfectant in aqueous solution using sodium thiosulphate pentahydrate and activated carbon.

Material and methods

As mentioned earlier, after completion of each bath treatment, treated seawater (seawater plus chemical disinfectant) was poured into a plastic bucket for the neutralisation treatment. Marine activated carbon (Aqua One Premium Carb) and/or sodium thiosulphate pentahydrate (Ace Chemical Company) were used as neutralisers. Firstly, marine activated carbon was added to the bucket and left acting for 20 minutes. Then, it was removed and sodium thiosulphate pentahydrate was added to the bucket and left acting for 5 minutes. The quantity of sodium thiosulphate pentahydrate and activated carbon used was different among the bath treatments. The levels of residual chemicals (benzalkonium chloride, free chlorine, iodine, chlorine dioxide and hydrogen peroxide) were measured several times using test strips which details are described in the 'Material and methods' of Part 1. Seawater temperature, pH and oxygen saturation were measured and recorded prior, during and after the neutralisation treatments.

Results

Treated seawater with the non-ionic surfactant EnviroClean at concentration of 1 %

Activated carbon (292 grams) did not decrease the level of residual benzalkonium chloride (around 250 ppm) in the seawater. Seawater temperature was on average 12.2 ± 0.3 °C and pH was 7. Activated carbon dropped oxygen saturation from 96.8 % to 72.5 %.

Treated seawater with calcium hypochlorite at concentration of 15 ppm

Activated carbon (146 grams) slightly decreased the level of residual free chlorine in the seawater. Then, sodium thiosulphate pentahydrate (230 milligrams) was added and decreased this level from around 10 ppm to 0 ppm. Seawater temperature was on average 12.2 ± 0.3 °C. Activated carbon dropped oxygen saturation from 96.0 % to 90.3 %. Sodium thiosulphate pentahydrate increased pH from 6 to 8.

Treated seawater with calcium hypochlorite at concentration of 25 ppm

Activated carbon (292 grams) slightly decreased the level of residual free chlorine in the seawater. Then, sodium thiosulphate pentahydrate (390 milligrams) was added and decreased this level from around 17 ppm to 0 ppm. Seawater temperature was on average 12.1 ± 0.1 °C. Activated carbon dropped oxygen saturation from 97.7 % to 88.8 %. Sodium thiosulphate pentahydrate increased pH from 6 to 8.5.

Treated seawater with the iodophor Buffodine at concentration of 50 ppm

Activated carbon (146 grams) slightly decreased the level of residual iodine in the seawater. Then, sodium thiosulphate pentahydrate (7 milligrams) was added and decreased the level of residual iodine from around 1 ppm to zero. Seawater temperature was on average 11.5 ± 0.1 °C and pH was 8. Activated carbon dropped oxygen saturation from 96.6 % to 90.0 %.

Treated seawater with the iodophor Buffodine at concentration of 150 ppm

Activated carbon (146 grams) slightly decreased the level of residual iodine in the seawater. Then, sodium thiosulphate pentahydrate (20 milligrams) was added and decreased the level of residual iodine from around 3 ppm to zero. Seawater temperature was on average 12.3 ± 0.1 °C and pH was 8. Activated carbon dropped oxygen saturation from 96.4 % to 90.2 %.

Treated seawater with the iodophor Buffodine at concentration of 250 ppm

Activated carbon (146 grams) slightly decreased the level of residual iodine in the seawater. Then, sodium thiosulphate pentahydrate (32 milligrams) was added and decreased the level of residual iodine from around 5 ppm to zero. Seawater temperature was on average 12.9 ± 0.1 °C and pH was 8.5. Activated carbon dropped oxygen saturation from 97.2 % to 93.1 %.

Treated seawater with the iodophor Ovadine at concentration of 50 ppm

Activated carbon (146 grams) slightly decreased the level of residual iodine in the seawater. Then, sodium thiosulphate pentahydrate (4 milligrams) was added and decreased the level of residual iodine from around 0.5 ppm to zero. Seawater temperature was on average 12.7 ± 0.2 °C and pH was 8. Activated carbon dropped oxygen saturation from 93.3 % to 88.9 %.

Treated seawater with the iodophor Ovadine at concentration of 150 ppm

Activated carbon (146 grams) slightly decreased the level of residual iodine in the seawater. Then, sodium thiosulphate pentahydrate (10 milligrams) was added and decreased the level of residual iodine from around 1.5 ppm to zero. Seawater temperature was on average 12.7 ± 0.1 °C and pH was 8. Activated carbon dropped oxygen saturation from 96.7 % to 92.1 %.

Treated seawater with the chlorine dioxide Zydox at concentration of 25 ppm

Activated carbon (292 grams) decreased the level of residual chlorine dioxide from around 25 ppm to 10 ppm in the seawater. Then, sodium thiosulphate pentahydrate (100 milligrams) was added and decreased this level to 0 ppm. Seawater temperature was on average 11.8 ± 0.1 °C and pH was 6. Activated carbon dropped oxygen saturation from 97.2 % to 79.8 %.

Treated seawater with the chlorine dioxide Zydox at concentration of 50 ppm

Activated carbon (292 grams) decreased the level of residual chlorine dioxide from around 50 ppm to 25–50 ppm in the seawater. Then, sodium thiosulphate pentahydrate (300 milligrams) was added and decreased this level to 0 ppm. Seawater temperature was on average 12.6 ± 0.1 °C. Activated carbon dropped oxygen saturation from 99.0 % to 92.4 %. Sodium thiosulphate pentahydrate increased pH from 6 to 8.

Treated seawater with hydrogen peroxide 50 % at concentration of 25 ppm

Activated carbon (292 grams) did not decrease the level of residual hydrogen peroxide (25 ppm) in the seawater. Seawater temperature was on average 12.8 ± 0.1 °C and pH was 8. Activated carbon dropped oxygen saturation from 98.5 % to 92.0 %.

Treated seawater with hydrogen peroxide 50 % at concentration of 50 ppm

Activated carbon (292 grams) did not decrease the level of residual hydrogen peroxide (50 ppm) in the seawater. Seawater temperature was on average 12.9 ± 0.1 °C and pH was 8. Activated carbon dropped oxygen saturation from 99.8 % to 90.4 %.

Discussion and future research

Bath treatment and monitoring period

A summary of the results is shown in Table 2. Changes in abalone behaviour were only observed during the bath treatments (Table 2). The strongest changes in behaviour were recorded during the bath treatment with the non-ionic surfactant *EnviroClean* at

concentration of 1 %. These changes include rapid movement of the foot, elevation of the shell and rotation of the shell around the muscle axis. These changes in abalone behaviour were significantly less strong during the bath treatments with calcium hypochlorite at concentrations of 15 and 25 ppm and with the chlorine dioxide *Zydox* at concentration of 50 ppm. Slow movement of the foot and elevation of the shell were observed during the bath treatments with the chlorine dioxide *Zydox* at concentration of 25 ppm and with the iodophor *Buffodine* at concentration of 250 ppm. The other bath treatments did not cause changes in abalone behaviour. Rapid movement of the foot, elevation of the shell and rotation of the shell around the muscle axis can also be observed in abalone when anaesthetic is added to tanks during stock movements on farm (Burke et al., 2001; Hooper et al., 2011).

Table 2

Summary of the results of the bath treatments and monitoring period. The results include changes in abalone behaviour, loss of abalone foot adhesion to the tub surface, number of moribund abalone and number of mortality (Y = yes; N = no).

Chemical disinfectant / Concentration	Bath treatment (60 minutes)			Monitoring period (15 days)			
	Change in behaviour	Loss of adhesion	Number of mortality	Change in behaviour	Loss of adhesion	Number of moribund	Number of mortality
Non-ionic surfactant *EnviroClean* 1%	Y	Y	0	N	Y	0	8
Calcium hypochlorite 15 ppm	Y	Y	0	N	N	0	0
Calcium hypochlorite 25 ppm	Y	Y	0	N	Y	2	0
Iodophor *Buffodine* 50 ppm	N	N	0	N	Y	1	0
Iodophor *Buffodine* 150 ppm	N	Y	0	N	N	0	0
Iodophor *Buffodine* 250 ppm	Y	Y	0	N	Y	0	0
Iodophor *Ovadine* 50 ppm	N	N	0	N	Y	1	0
Iodophor *Ovadine* 150 ppm	N	N	0	N	Y	0	0
Chlorine dioxide *Zydox* 25 ppm	Y	Y	0	N	Y	3	0
Chlorine dioxide *Zydox* 50 ppm	Y	Y	0	N	Y	4	1
Hydrogen peroxide 50% 25 ppm	N	N	0	N	Y	2	0
Hydrogen peroxide 50% 50 ppm	N	Y	0	N	Y	0	0

When combining the results from the bath treatments and the monitoring period, all chemical disinfectants caused abalone to lose their foot adhesion to the tub surface (Table 2). Moribund abalone and mortality were only recorded during the monitoring period (Table 2). The bath treatments with the chlorine dioxide *Zydox* at concentrations of 25 and 50 ppm caused the highest number of moribund abalone during the monitoring period (n= 3 and 4, respectively), and the latter caused one mortality. All abalone from the bath treatment with the non-ionic surfactant *EnviroClean* at concentration of 1 % were dead on the first day of the monitoring period.

Other chemical product that can cause stress to abalone and mortality is anaesthetic (Burke et al., 2001; Hooper et al., 2011). For example, the effects of benzocaine anaesthetic on

abalone haemolymph included acute depression of phagocytosis, antibacterial activity and lysosomal neutral red retention time, as well as elevated haemocyte density (Hooper et al., 2011). Benzocaine anaesthetic also caused histological changes in tissues of abalone (Hooper et al., 2014). Based on this result, it is expected that the chemical disinfectants used in this trial caused histological changes in the tissues of the abalone. Therefore, the abalone that were fixed in formalin at the end of the monitoring period, which includes some moribund abalone, should be submitted for histological analysis. For future trials, collection of abalone blood samples for biochemistry and immunological analysis should be considered.

Chemical residues on abalone tissues can occur as a result of bath treatments and must be investigated. Concern for the presence of chemical residues in edible tissues is associated with possible effects on consumer health such as immediate hypersensitivity reactions, effects on gut flora and potential toxicological effects (GESAMP, 1997). In order to ensure that no residues above maximum residue limits are present in edible tissues of aquaculture products, a withholding period is introduced following treatment (i.e. a time delay that is imposed between end of treatment and harvesting for human consumption) (GESAMP, 1997). For example, a withholding period of 500 degree days (50 days at 10 °C water temperature or 25 days at 20 °C water temperature) is required for the residues to be 'eliminated' from abalone tissues after a treatment with benzocaine anaesthetic (APVMA Permit 14289). Note that, residue clearance (withholding period) can cause harvesting delay for abalone close to market size and can generate costs with residue testing. Because this chemical residue matter is of significant importance, the abalone that were frozen at the end of the monitoring period in this trial should be submitted for residue testing to assess the levels of residual chemicals on the abalone tissues.

In the present trial, the levels of residual chemicals (benzalkonium chloride, free chlorine, iodine, chlorine dioxide and hydrogen peroxide) in the seawater did not decrease significantly from the beginning to the end of the bath treatments (60 minutes). This result could be attributed to the filtered seawater (to 1 µm) used in this trial. It is well known that organic matter can inactivate a number of chemical disinfectants (Department of Agriculture Fisheries and Forestry, 2008). According to Department of Agriculture Fisheries and Forestry (2008), chlorine has high loss of activity in the presence of organic matter, iodine has moderate to high loss of activity and chlorine dioxide has low to moderate loss of activity. Other consideration is the gradations of the test strips used in this trial to measure the levels of residual chemicals. For example, the test strip used to measure the level of residual chlorine dioxide has gradation of 0, 10, 25, 50, 100, 250, 500 ppm, so a result of 25

ppm may not accurately be 25 ppm. Therefore, titration methods and/or portable meters should be used in future trials to improve accuracy of the results.

Seawater temperature was constant during the bath treatments and the monitoring period. Hydrogen peroxide 50 % at concentration of 50 ppm increased oxygen saturation slightly from 98.4 to 100.4 % during the bath treatment. Oxygen saturation should be monitored when using hydrogen peroxide as oxygen saturation in excess of 115 % can increase the incidence of gas bubble disease, bacterial infection and subsequent mortality in abalone (Elston 1983; Elston and Lockwood 1983). Regarding pH, calcium hypochlorite at concentrations of 15 and 25 ppm and the chlorine dioxide *Zydox* at concentrations of 25 and 50 ppm dropped pH from 8 to 6 during the bath treatments. pH should be monitored when using these chemicals as greenlip abalone mortality was significantly increased at pH 6.79 (Harris et al., 1999a). Although the pH 6 recorded in this trial did not cause mortality during the bath treatments (60 minutes), it probably increased stress on the abalone.

Other considerations when administering treatments with chemical disinfectants are (Department of Agriculture Fisheries and Forestry, 2008):

- Accumulations of soil, dirt or organic matter provide an effective barrier, protecting pathogens from disinfecting agents;
- The effectiveness of certain disinfectants depends on the quality and hardness of the water used;
- The effectiveness of chemical disinfectants depends on the concentration used, as well as the contact time;
- The effectiveness of many chemical disinfectants depends on temperature and pH;
- Many disinfectants are corrosive to equipment, and most are irritant to people and toxic to aquatic life.

Future on farm trials should be undertaken once more information is gathered. The effectiveness of the chemical disinfectants used in this trial against *Haliotid herpesvirus 1* (HaHV) in seawater containing organic matter need to be investigated. Their initial concentrations, residual levels and exposure times need to be determined. Because this research involves HaHV, it cannot be undertaken on farm. Yumbah Aquaculture should collaborate with Commonwealth Scientific and Industrial Research Organisation (CSIRO) to develop this research at the Australian Animal Health Laboratory in Geelong, Victoria. Other valuable research is to develop methods to detect and possibly measure HaHV in seawater. Detection of *Ostreid herpesvirus 1* (OsHV-1) in seawater was studied by Evans et al. (2014 and 2017). It may be possible to adapt their method to detect HaHV.

Neutralisation treatment

As expected, sodium thiosulphate pentahydrate neutralized the residual free chlorine, iodine and chlorine dioxide in treated seawater. It increased pH from 6 to 8 in treated seawater with calcium hypochlorite and chlorine dioxide. Marine activated carbon worked best in treated seawater with the chlorine dioxide *Zydox*, decreasing significantly the level of residue. It slightly decreased the levels of residual free chlorine and iodine in treated seawater, and did not change the levels of residual benzalkonium chloride and hydrogen peroxide in treated seawater. Residual benzalkonium chloride was removed in water by activated carbon during Tanada et al. (1991) study. The quantity of marine activated carbon used in this trial and contact time may need to be increased to improve its effectiveness. Kubota et al. (2013) found that activated carbon removed iodine in freshwater but hardly removed iodine in seawater. Regarding hydrogen peroxide, Jones (2007) recommends the use of ferrous sulphate ($FeSO_4$) to neutralize hydrogen peroxide in aqueous solution. Marine activated carbon significantly decreased oxygen saturation in all neutralisation treatments during this trial. Oxygen saturation should be monitored when using activated carbon as greenlip abalone mortality was significantly increased at 65 % or lower (Harris et al., 1999b).

Commented [C3]: Hazardous or non-hazardous???

As mentioned earlier, titration methods and/or portable meters should be used in future trials to improve accuracy of the results of the levels of residual chemicals in seawater. A valuable trial is to assess the effects of the residual chemicals (free chlorine, iodine, chlorine dioxide and hydrogen peroxide) on biofilter and their neutralisation treatments in a recirculating system on farm.

Commented [C4]: It should contain abalone. Need to test the chemical neutralizer on abalone. I didn't do that.

Conclusion

Based on the results of the present trial, the non-ionic surfactant *EnviroClean* should not be tested in future trials as it caused 100 % mortality. The chlorine dioxide *Zydox* should be tested in future trials but at lower concentrations and/or shorter exposure time. Calcium hypochlorite, hydrogen peroxide and the iodophors *Buffodine* and *Ovadine* should be also tested in future trials. As mentioned earlier, fixed abalone samples should be submitted for histological analysis and frozen abalone samples for residue testing. The results should be included in this report and they may affect the conclusions.

Some considerations from the information cited in this report are:

- Calcium hypochlorite and *Buffodine* can inactivate *Haliotid herpesvirus 1* (HaHV), but there is no information on the other chemicals against this virus;
- The chemical has to be approved by APVMA to be used on abalone. At the moment, there is an APVMA permit (PER83276) for hydrogen peroxide 60 % to be used in the treatment of metazoan and protozoan ecto-parasitic infestations and the control of

fungal infections in freshwater and saltwater finfish and finfish eggs. The bath treatment can be up to 60 minutes and the withholding period is nil;

- A chemical with short or no withholding period is desirable;
- Organic matter inactivates a number of chemical disinfectants;
- Hydrogen peroxide can harm biofilter in a recirculating aquaculture system;
- *Ovadine* is more environment-friendly than *Buffodine* because the latter has a non-ionic surfactant in its composition.

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