International Journal for Parasitology 42 (2012) 1135–1141

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Seaweed extracts as a natural control against the monogenean ectoparasite, *Neobenedenia* sp., infecting farmed barramundi (*Lates calcarifer*)

Kate S. Hutson *, Leonardo Mata, Nicholas A. Paul, Rocky de Nys

Centre for Sustainable Tropical Fisheries and Aquaculture, School of Marine and Tropical Biology, James Cook University, Townsville, Queensland 4811, Australia

ARTICLE INFO

Article history:
Received 13 August 2012
Received in revised form 20 September 2012
Accepted 21 September 2012
Available online 12 October 2012

Keywords:
Anthelmintic
Aquaculture
Seaweed
Monogenea
Parasite management
Natural treatments
Asian sea bass
Lates calcarifer

ABSTRACT

Aqueous extracts from common tropical seaweeds were evaluated for their effect on the life cycle of the commercially important ectoparasite, *Neobenedenia* sp. (Platyhelminthes: Monogenea), through the survival of attached adult parasites, period of embryonic development, hatching success and oncomiracidia (larvae) infection success. There was no significant effect of any extract on the survival of adult parasites attached to fish hosts or infection success by oncomiracidia. However, the extracts of two seaweeds, *Ulva* sp. and *Asparagopsis taxiformis*, delayed embryonic development and inhibited egg hatching. The extract of *A. taxiformis* was most effective, inhibiting embryonic development of *Neobenedenia* sp. and reducing hatching success to 3% compared with 99% for the seawater control. Furthermore, of the 3% of eggs that hatched, time to first and last hatch was delayed (days 14 and 18) compared with the seawater control (days 5 and 7). *Asparagopsis taxiformis* shows the most potential for development as a natural treatment to manage monogenean infections in intensive aquaculture with the greatest impact at the embryo stage.

1. Introduction

Parasite infections are an important animal health and management consideration in aquaculture with persistent disease impacting whole industry sectors. Of the diversity of parasites that impact aquaculture industries, monogeneans (flatworms) of the genus *Neobenedenia* (Platyhelminthes: Monogenea) are known pathogens of tropical and subtropical fishes in marine aquaria and aquaculture (Bondad-Reantaso et al., 1995; Ogawa et al., 2006; Hirayama et al., 2009; Whittington, 2012). This includes the important aquaculture species, Asian sea bass or barramundi (*Lates calcarifer*), in Indonesia (Rückert et al., 2008) and Australia (Deveney et al., 2001) where *Neobenedenia* infections are associated with significant fish losses. For example, estimates of total *L. calcarifer* losses resulting from a single outbreak of *Neobenedenia* in Queensland, Australia, were 200,000 fish (~50 tonnes; worth AUD $500,000) (Deveney et al., 2001).

Neobenedenia are obligate parasites with a direct life cycle. Juvenile and adult parasites attach to the skin, fins and eyes of fish using a sucker-like haptor and graze on host epidermis (Ogawa et al., 1995, 2006). Adult parasites lay eggs into the water column, however filaments on the eggs entangle in net cages and are retained within the culture environment (Kearn et al., 1992). Eggs then hatch into free-swimming, ciliated oncomiracidia that directly re-infect fish. High infection intensities result in damage to the epidermis that facilitates infections by bacteria, fungi or viruses (Thoney and Hargis, 1991).

There are no effective methods to prevent *Neobenedenia* infections in open aquaculture systems and the current parasite management effort only allows temporary respite by removing attached parasite stages (Whittington, 2012). As a treatment, infected fish are typically immersed or 'bathed' in hydrogen peroxide or formalin solutions, or in freshwater (Ernst et al., 2002; Ogawa et al., 2006). These treatments are often ineffective in killing developing embryos within eggs (Sharp et al., 2004; Fajer-Ávila et al., 2007). Treatments are also labour-intensive and stressful to fish, and mortalities may occur due to difficulties in calculating bath solution concentrations, physical damage to fish from crowding or lack of oxygen (Williams et al., 2007). Although bath treatments are effective at killing adult monogeneans, re-infection from untreated eggs and larvae in the culture environment occurs immediately following treatment. In closed systems such as brood stock facilities, land-based nurseries and public aquaria, effective parasite control also requires methods to eliminate viable eggs from tanks and equipment (Ernst et al., 2005).

Natural (or "green") treatments are emerging as an alternative approach to control bacterial, viral and fungal pathogens in aquaculture (Lio-Po et al., 2005; Genovese et al., 2012; Selvin et al., 2011; Sudheer et al., 2011) and within this framework, compounds

* Corresponding author. Tel.: +61 7 47816216; fax: +61 7 47814585.
E-mail address: kate.hutson@jcu.edu.au (K.S. Hutson).

0020-7519/$36.00
http://dx.doi.org/10.1016/j.ijpara.2012.09.007

isolated from marine algae (seaweeds) have potential to control parasites. Seaweeds are a rich source of biologically active natural products with properties ranging from antibacterial to anti-inflammatory and anthelmintic (see Smit, 2004; Blunt et al., 2012 for reviews). We hypothesise that algal natural products can control metazoan parasite infections of fishes as an underlying basis for natural treatments. Furthermore, seaweeds can be cultivated in proximity to, or integrated within, finfish aquaculture systems with benefits for both resources (Chopin et al., 2001; Troell, 2009; Mata et al., 2010). Water-soluble natural products are specifically targeted through selective extraction, as this methodology negates the use of organic solvents and can be directly implemented with basic technologies (McCloud, 2010). However, no studies have examined the potential impact of either live seaweeds, or natural products released from seaweeds, on ectoparasites. Therefore, the aim of this research was to determine the effects of water-soluble natural products (extracts) from a range of tropical seaweeds across egg, larval and adult life stages of the ectoparasite, *Neobenedenia* sp., infecting *L. calcarifer*.

2. Materials and methods

2.1. Source and identification of monogenean parasites

Neobenedenia sp. used in experiments were collected from two separate localities. The first collection was from hatchery-reared juvenile barramundi, *L. calcarifer*, maintained in seawater (35‰) in the Marine Aquaculture Research Facility Unit (MARFU) at James Cook University, Australia (19°19′42″S 146°45′40″E). The original source of infection in hatchery-reared fish is unknown. Parasites from this collection were used in experiments described in Sections 2.3 and 2.4. A laboratory infection was also established from embryonated *Neobenedenia* sp. eggs collected from a land-based marine *L. calcarifer* farm, Good Fortune Bay Fisheries Ltd., Queensland, Australia (19°56′24″S 147°55′54″E), in June 2011. Eggs were introduced to laboratory fish (supplied by Mainstream Aquaculture Ltd., Australia) held in 100 L aquaria in the laboratory and maintained at 35‰. Parasites from the laboratory infection were used in experiments described in Sections 2.5 and 2.6.

The species of *Neobenedenia* investigated in this study is unidentified. Although *Neobenedenia melleni* was previously identified from Queensland (Deveney et al., 2001) it is now considered to be a species complex due to its geographic distribution, host associations, biology and taxonomy (Whittington, 2004). *Neobenedenia girellae* and *Neobenedenia parguerae*nsis are also considered to be synonyms of *N. melleni* (see Dyer et al., 1992; Whittington and Horton, 1996). Representative specimens from the two populations sampled were removed from fish by immersing them in dechlorinated freshwater for 5 min. Parasites were collected from the solution and fixed in 100% absolute ethanol. Fixed parasites were placed in distilled water before being stained in Mayer's haematoxylin and then destained in 1% HCl in 70% ethanol. Specimens were dehydrated in an ethanol series before being cleared in cedar wood oil and mounted on a slide in Canada balsam. Parasites collected from the two localities were morphologically similar and identified as *Neobenedenia* sp. (hereafter as *Neobenedenia*) by Ian D. Whittington, South Australian Museum, Australia (SAMA), and accessioned in the Australian Helminth Collection (AHC); SAMA AHC 35240–41 ex MARFU, Townsville and SAMA AHC 35461 ex Good Fortune Bay, Bowen, Australia.

2.2. Seaweed extracts

Eight seaweed species were selected to test the activity of extracts on stages of the *Neobenedenia* life cycle based on their potential to be cultivated in tanks, raceways and ponds, and to be integrated with fish aquaculture systems. These are the red seaweeds, *Asparagopsis taxiformis*, *Gracilaria edulis* and *Hypnea musciformis*; the green seaweeds, *Caulerpa taxifolia*, *Derbesia tenuissima* and *Ulva* sp.; and the brown seaweeds, *Cystoseira trinodis* and *Dictyopteris delicatula*. *Cystoseira trinodis* extract was toxic to fish (see Section 2.3) and not used in further experimentation, while *H. musciformis* was unavailable during experiments with eggs and replaced by the red seaweed *Halymenia floresii* (see Section 2.4). Each seaweed species was held or cultured in individual tanks in a recirculation aquaculture system at MARFU prior to extraction. Fresh seaweed was spun dry in a fine mesh bag before weighing. Water-soluble natural products were extracted by blending (Abode Appliances, model No. YD-2198) each species in filtered seawater for 2 min at a wet weight to volume ratio of 0.1 g/ml. Each solution was then poured into a Schott bottle and placed on a shaking table at room temperature for 4 h to allow mixing. Each solution was sieved through a fine mesh cloth (60 μm) into centrifuge tubes, each spun at 5,300*g* for 5 min. The supernatant was then transferred to a clean Schott bottle and stored at 4 °C for no longer than 24 h before use. Extracts were freshly made for each experiment before use and discarded at the end of each experiment (maximum 21 days) to avoid any degradation or loss of natural products through storage.

2.3. Effect of seaweed extracts on in vivo Neobenedenia survival

The effect of seaweed extracts on the survival of attached adult *Neobenedenia* was tested in vivo. Ten replicate *L. calcarifer* infected with *Neobenedenia* were exposed to extracts of each seaweed species (*A. taxiformis*, *G. edulis*, *H. musciformis*, *C. taxifolia*, *D. tenuissima*, *Ulva* sp., *C. trinodis* and *D. delicatula*) at a concentration of 1 mL of extract to 100 mL of seawater, in addition to a UV filtered seawater control. This concentration was selected based on results of a pilot study that showed that adult parasites did not survive >24 h in diluted 1:10 mL or 1:100 mL concentrations of *A. taxiformis* extract in vitro (unpublished data). Experiments using attached adult parasites in this study were conducted in vivo. The effect of extracts on parasite survival was examined following 24 h exposure. *Lates calcarifer* were <1 year-old hatchery-reared juvenile barramundi maintained in a 2,500 L circular tank containing seawater in MARFU. Eighty barramundi were removed from the tank using a net and placed into individual 10 L containers with constant aeration. Ten replicates were used for extracts of each of the eight seaweed species plus 10 replicate controls in UV-filtered seawater. Each fish was maintained in 6 L of either treatment or control solution for 24 h at room temperature. Fish were not fed during this period and mortalities were noted and excluded from data analysis. *Cystoseira trinodis* extract was not included in the analyses due to 100% mortality of experimental fish.

Dead *Neobenedenia* were recovered from the treatment and control solutions by filtering through a 60 μm mesh. Dead parasites usually detach from the host surface and sink to the bottom of the container. In addition, to ensure all dead parasites had detached from the body of the fish, the external surface of each fish was gently rubbed by hand prior to filtering (Chambers and Ernst, 2005). Parasite specimens were collected in 250 mL jars and fixed in 70% ethanol. Fish were removed from treatment containers by hand and placed individually into 6 L of dechlorinated freshwater for 5 min to kill the remaining live *Neobenedenia* attached to fish. Live *Neobenedenia* are transparent, hence the only method to accurately quantify the total number infecting fish is to kill the parasites which renders them opaque and therefore visible to the naked eye. Fish were again gently rubbed by hand to ensure that all dead *Neobenedenia* had detached prior to returning the fish to a seawater recovery tank. The dechlorinated freshwater solution

was filtered through a 60 μm mesh, and parasites were collected in 250 mL jars and fixed in 70% ethanol. All parasite specimens were counted under a stereomicroscope.

2.4. Effect of seaweed extracts on time to first and last hatch and hatching success of Neobenedenia eggs

Neobenedenia eggs are a key life stage as they hatch to release swimming, infectious oncomiracidia. Seaweed extracts were tested for their effects on the time to first and last hatch and hatching success of *Neobenedenia* eggs. Adult *Neobenedenia* were dislodged from the body surface of three infected fish by carefully sliding a scalpel blade beneath the haptor of attached parasites. Parasites were then placed in a 200 mL plastic container in filtered seawater. The solution was poured off and replaced 1 h later, once live parasites had attached to the base of the container. Eggs laid into the seawater over the following 12 h period were carefully removed using a paintbrush. Eggs were examined under a dissecting microscope and a needle was used to allocate 10 to 12 eggs to each well of five 24–well culture plates (LOMB Scientific 92424 tissue culture plates) containing 3 ml of filtered seawater. Filtered seawater was then replaced by 3 ml of solution containing extracts of seaweeds (*A. taxiformis*, *G. edulis*, *H. floresii*, *C. taxifolia*, *D. tenuissima*, *Ulva* sp. and *D. delicatula*) diluted 1:100 mL, while control wells were replaced by new filtered seawater. Eggs were slightly negatively buoyant so that solutions could be exchanged with minimal exposure to air.

Culture plates were then incubated in culture chambers (Sanyo: ML-351 Versatile Environmental Incubation Chamber) at 22 °C (average winter sea surface temperature for northern Queensland) on a 12:12 h day:night cycle. Treatments were allocated in six wells on each of two plates ($n = 12$), and each plate had a seawater control in six wells ($n = 30$). Two millilitres of treatment and control solution were exchanged daily in each well by gentle pipetting to retain eggs within the wells. Plates were monitored daily at 15:00 h under a stereomicroscope for hatching of free-swimming oncomiracidia. Oncomiracidia were counted and removed by gentle pipetting under a dissection microscope at the end of each day and fixed in 100% absolute ethanol. The experiment was terminated once hatching ceased (48 h following no hatching in any treatment). The total number of eggs and the number of eggs with opened opercula (Fig. 1) were counted after hatching had ceased. Hatching success was quantified by the number of oncomiracidia recovered from the total number of eggs, and the number of eggs with opened opercula from the total number of eggs.

2.5. Effect of Ulva sp. and A. taxiformis extract on Neobenedenia embryo development, time to first and last hatch, and hatching success

Extract from two seaweeds, *Ulva* sp. and *A. taxiformis*, were active in the previous assay (see Section 3.2) and were therefore investigated in greater detail for their effects on embryo development, time to first and last hatch, and hatching success. Four *L. calcarifer* infected with *Neobenedenia* were removed from the laboratory infection and held overnight (12 h) in a 20 L container containing filtered seawater (35‰). Eggs were collected by filtering the seawater through a 60 μm mesh. Bundles of eggs were immediately placed into extracts of *Ulva* sp. (1:100 mL), *A. taxiformis* (1:100 mL) or a control (UV filtered sea water). Subsequently, eggs were carefully separated using a paintbrush and needle under a dissecting microscope and between 10 and 27 eggs were allocated to an individual glass cavity block (40 mm^2) with 12 replicates for each treatment, including the control. The cavity block was filled to the brim with the appropriate treatment or control solution and a glass lid was gently pushed across the surface so that no air was trapped beneath the lid. This precaution was taken because oncomiracidia can lyse or become trapped when contacting the surface tension of their container (Glennon et al., 2006). Two millilitres of treatment or control solution were exchanged daily in each well by gentle pipetting to prevent removal of eggs. Cavity blocks were incubated in culture chambers (Sanyo: ML-351 Versatile Environmental Incubation Chamber) at 22 °C on a 12:12 h day:night cycle.

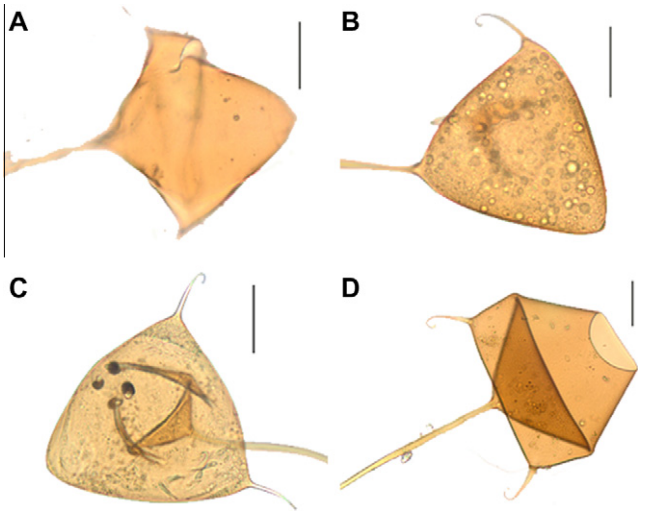

Fig. 1. *Neobenedenia* egg development. Egg development was scored as stage I = non-embryonated (A); stage II = embryonated (B); stage III = developing (C); and stage IV = hatched (D). Scale bars = 50 μm.

Eggs were monitored daily at 15:00 h under a stereomicroscope with both incident and transmitted light. Embryo development was scored according to four developmental stages: stage I = non-embryonated; eggs clear in colour and/or visibly damaged; stage II = embryonated; eggs dark brown in colour with evidence of embryos undergoing cellular division; stage III = developing; embryos with eye-spots present; and stage IV = hatched; eggs clear in colour with opened operculum (Fig. 1). The progress of embryo development was monitored daily using this methodology. Oncomiracidia were removed using a fine glass pipette and counted. The experiment was terminated when hatching ceased (72 h following no hatching in any treatment). The two hatching success parameters were reported as in Section 2.4.

2.6. Effect of Ulva sp. and A. taxiformis extract on infection success

Seaweed extracts were tested on the ability of oncomiracidia to successfully locate, infect and develop on host fish under experimental conditions. *Lates calcarifer* used in infection experiments were <1 year-old, mean 110 mm L_T (85–126 mm) freshwater hatchery-reared fish from Good Fortune Bay Ltd., Kelso, Townsville, Australia (19°21′25″S 147°42′20″E). Twenty-four fish were acclimated to seawater over 2 h. Subsequently, individual fish were gently netted into a 10 L container with 3 L of filtered seawater (35‰) and held for 24 h prior to experiments in an air-conditioned room (25 °C) exposed to natural light and constant aeration. Eight randomly assigned replicates were used for extracts of each of the two seaweed species, *Ulva* sp. and *A. taxiformis*, (1 ml seaweed extract: 100 mL filtered seawater) and eight replicate controls in UV filtered seawater (1 ml filtered seawater: 100 mL filtered seawater). Seven vigorously swimming oncomiracidia (derived from eggs collected as in Section 2.5) incubated in 35‰ filtered seawater in a 25 °C air-conditioned room, were gently pipetted into each replicate container using a glass pipette, following the addition of the extract. Fish were held for 60 h to allow oncomiracidia to attach to their host and commence development. Fish were not fed during

this period and mortalities were noted and excluded from data analysis. Individual fish were removed from the container by hand and placed in dechlorinated freshwater for 5 min to kill attached juvenile *Neobenedenia*. The external surface of each fish was gently rubbed by hand prior to examining the freshwater solutions under a stereomicroscope. Infection success was quantified by the number of juvenile *Neobenedenia* recovered from the total number of oncomiracidia.

2.7. Statistical analysis

All analyses were done using the permutational multivariate analysis of variance (PERMANOVA + Version 1.0.3) add-on for PRIMER 6 (Version 6.1.13). Hatching success of oncomiracidia over time was analysed using Bray-Curtis similarities of the frequency of oncomiracidia each day, whereas the overall hatching success of oncomiracidia (as a percentage of available eggs) was analysed separately. Post-hoc comparisons were made using pair-wise combinations of treatments.

3. Results

3.1. Effect of seaweed extract on in vivo survival of Neobenedenia

Seaweed extracts did not kill attached adult *Neobenedenia*. All *Neobenedenia* survived in all seaweed extracts and the seawater control with the exception of three individuals (one in each of three replicates) in the extract of *A. taxiformis*, and a single specimen in the extract of *D. delicatula* (Fig. 2A). Freshwater baths confirmed that 99% of experimental fish were infected with *Neobenedenia* at the commencement of the experiment, with a mean infection intensity of 9.6 ± 0.7 SE parasites per fish (Fig. 2B). All infected fish were host to adult *Neobenedenia*, with eggs produced over the 24 h period present in treatment and control solutions. *Cystoseira trinodis* extract was toxic to fish causing 100% mortality, while a single mortality occurred in extracts of *C. taxifolia*, *D. tenuissima*, *D. delicatula*, *H. musciformis* and *Ulva* sp.

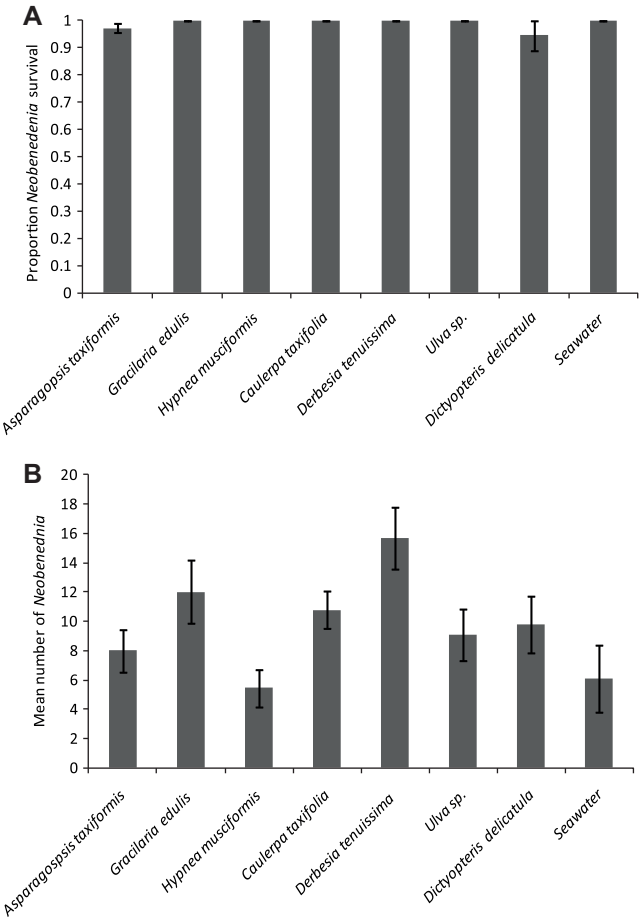

Fig. 2. Mean proportion of *Neobenedenia* that survived in vivo in seaweed treatments and the seawater control (A) and mean number of *Neobenedenia* infecting *Lates calcarifer* in each treatment at the commencement of the experiment (B). Error bars indicate SE.

3.2. Effect of seaweed extracts on time to first and last hatch and hatching success of Neobenedenia eggs

Seaweed extracts had differential effects on the hatching success of oncomiracidia (PERMANOVA, Pseudo-$F_{7,106}$ = 12.94, P = 0.001; Fig. 3), which resulted in three groupings of treatments that could be broadly classified as: (i) short embryonation period (6–8 days) and high hatching success (>60%; including the control, *G. edulis* and *H. floresii*); (ii) short embryonation period (6–7 days) and low hatching success (<60%; including *C. taxifolia* and *D. tenuissima*); and (iii) prolonged embryonation period (>8 days) and low hatching success (<60%; including *D. delicatula*, *Ulva* sp. and *A. taxiformis*) (Fig. 3). To a large extent, overall hatching success reflected the embryonation timeframes (PERMANOVA, Pseudo-$F_{7,103}$ = 6.99, P = 0.001), separating *Ulva* sp. and *A. taxiformis* from all other treatments, with the exception of high hatching success treatments of *G. edulis* and *H. floresii* being greater also than *D. tenuissima*, the third lowest overall success (Fig. 3).

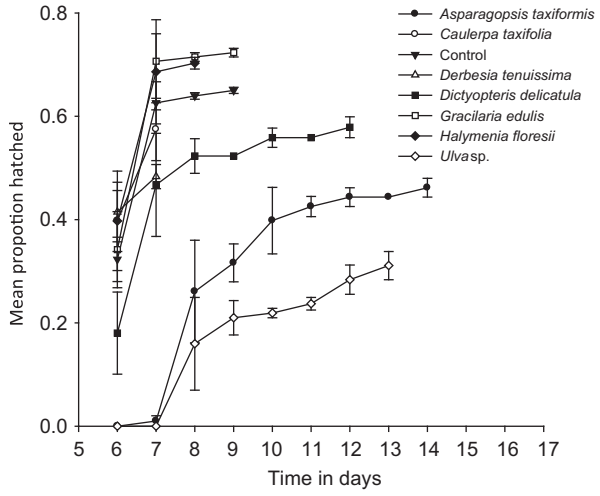

Fig. 3. Cumulative daily proportion of successful *Neobenedenia* hatching as evidenced by oncomiracidia removed. Day of last hatch indicated for each treatment where data points finish. Error bars indicate SE.

3.3. Effect of Ulva sp. and A. taxiformis extract on Neobenedenia embryo development, time to first and last hatch, and hatching success

Asparagopsis taxiformis extract inhibited embryo development, delayed time to first and last hatch, and significantly inhibited egg hatching success. The ontogeny of egg development could be differentiated between the treatments. The control and *Ulva* sp. treatments behaved similarly through to oncomiracidia recovery, with the extract of *A. taxiformis* clearly limiting development of the embryos (Fig. 4, PERMANOVA, Pseudo-$F_{2,33}$ = 91.74, P = 0.001; Control = *Ulva* sp. > *A. taxiformis*). A small proportion of eggs was not embryonated (Stage 1; Fig. 4). Eggs in the extracts of *A.*

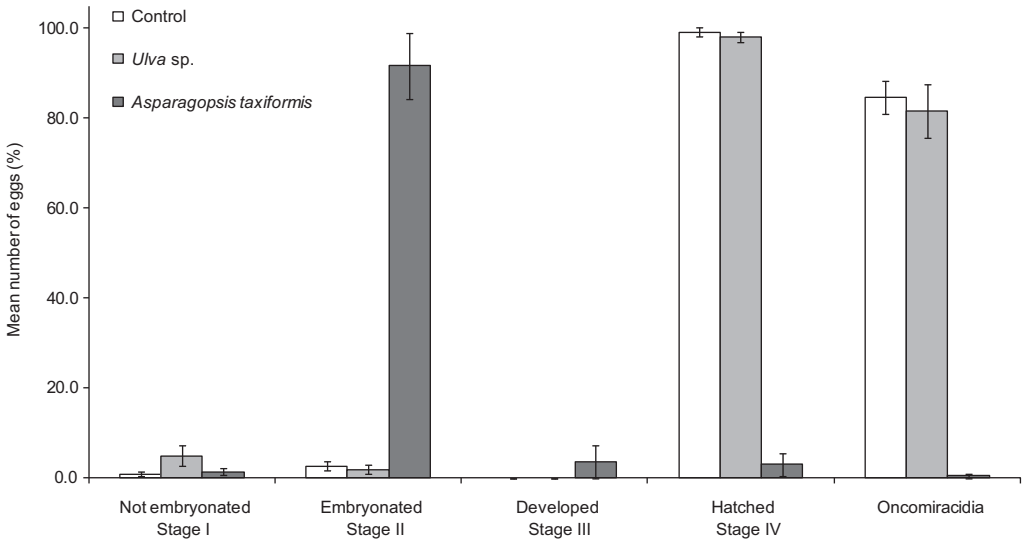

Fig. 4. Mean percentage of *Neobenedenia* eggs at four stages of egg development and mean percentage of oncomiracidia removed, in *Ulva* sp. and *Asparagopsis taxiformis* extract, and in the seawater control following 21 days of exposure. Error bars indicate SE.

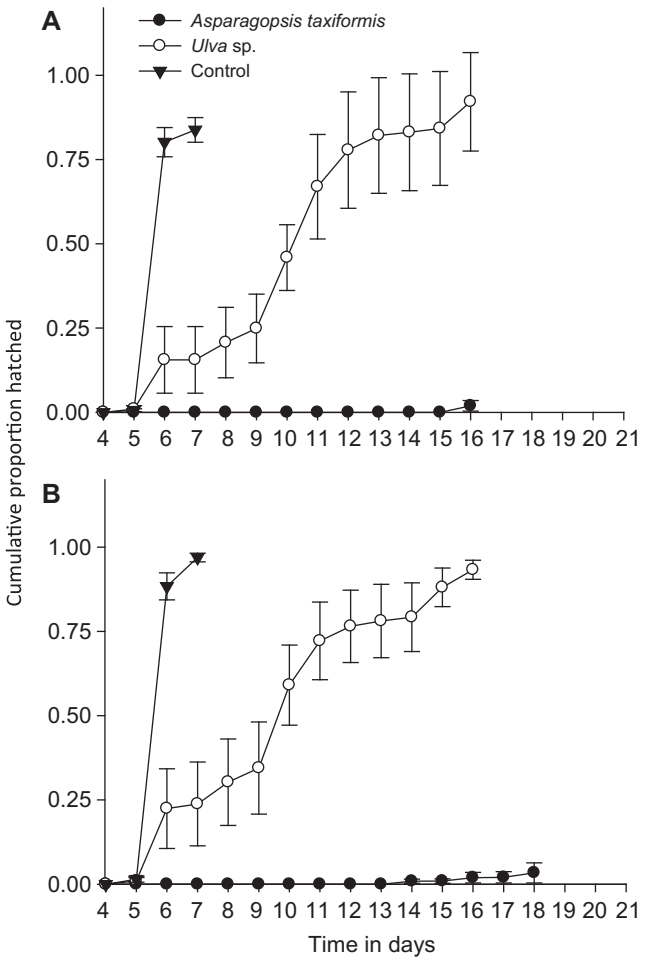

Fig. 5. Cumulative proportion of hatched *Neobenedenia* as evidenced by oncomiracidia (A) and Stage IV hatched eggs (B) (see Fig. 1) incubated in *Ulva* sp., *Asparagopsis taxiformis* extracts and in seawater controls. Day of last hatch is indicated for each treatment where data points finish. Error bars indicate SE.

taxiformis were embryonated, but the majority (92%) failed to develop eye-spots (Fig. 4). A small proportion of eggs which developed eye-spots in the extract of *A. taxiformis* (4%, Fig 4; Stage III, Fig. 1) failed to hatch (Fig. 4).

Time to first and last hatch differed between *Ulva* sp. and the control, and *A. taxiformis*, over the 18 days (Fig. 5A, PERMANOVA, Pseudo-$F_{2,33}$ = 15.86, P = 0.001; all pairwise comparisons < 0.05). Eggs in seawater controls began hatching on day 5 with no hatching following day 7 (Fig. 5A), while extract of *Ulva* sp. delayed time to first and last hatch with oncomiracidia hatching from day 5 to 16, although overall hatching success was high (98%; Fig. 5A). All oncomiracidia in *A. taxiformis* hatched on day 16, however hatching success was very low. Overall oncomiracidia hatching success (the number of oncomiracidia removed) was reduced to <1% in *A. taxiformis* compared with *Ulva* sp. (86%) and the seawater control (76%) (Fig. 5A, PERMANOVA, Pseudo-$F_{1,15}$ = 215.16, P = 0.001; Control = *Ulva* sp. > *A. taxiformis*).

Hatching success, as quantified by Stage IV eggs, reflected the same outcome with 99%, 98% and 3% hatching for the seawater control and extracts of *Ulva* sp. and *A. taxiformis*, respectively (Fig. 5B). After hatching ceased, the total number of eggs with opened opercula was counted, confirming that 84% of the emerging oncomiracidia were removed during the experiment and indicating that some oncomiracidia may have lysed (Fig. 4). However, the number of oncomiracidia that lysed was low and does not limit our interpretations (Fig. 5A, B).

3.4. Effect of Ulva sp. and A. taxiformis extract on infection success of L. calcarifer

Oncomiracidia infection success was lower in extracts of *A. taxiformis* (51%) and *Ulva* sp. (54%) compared with seawater controls (71%; Fig. 6). However, infection success in the presence of the seaweed extracts was not significantly different to that of the control (Fig. 6; PERMANOVA, Pseudo-$F_{2,33}$ = 1.40, P = 0.302). Oncomiracidia successfully infected *L. calcarifer* in all replicates in all treatments. A single mortality of *L. calcarifer* occurred in extract of *Ulva* sp. and two mortalities occurred in extract of *A. taxiformis*. No mortalities were recorded in the seawater control.

4. Discussion

Extracts containing water-soluble natural products from some seaweeds inhibited embryonic development, delayed time to first

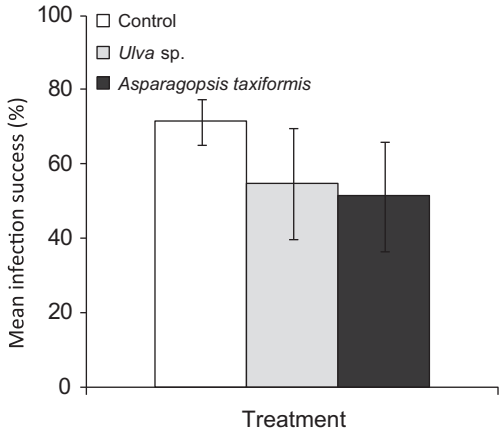

Fig. 6. Mean percentage of *Neobenedenia* infection success of *Lates calcarifer* exposed to *Ulva* sp. and *Asparagopsis taxiformis* extracts and the seawater control. Error bars indicate SE.

and last hatch, and reduced hatching success of the ectoparasite monogenean, *Neobenedenia*. This clearly identifies a mechanism to manage some aspects of the life cycle of *Neobenedenia* in cultured fishes with the integration of innovative natural treatments. These treatments would specifically be used to target parasite eggs to break life cycles, as seaweed extracts did not affect attached adult parasite stages or prevent oncomiracidia locating and infecting their host fish. The water-soluble extract of the red seaweed *A. taxiformis* had the strongest effect, inhibiting larval development inside *Neobenedenia* eggs, and almost entirely preventing hatching (3% hatching success; Figs. 4 and 5). Ethanol extracts of *A. taxiformis* have broad biological activity with antibacterial properties against pathogenic bacteria in aquaculture (Genovese et al., 2012) and in human pathogens (Paul et al., 2006b). Importantly, the natural products produced by *Asparagopsis* spp. (*A. taxiformis* and *Asparagopsis armata*; halogenated alkanes, alkenes, alkynes and acrylic acids) are well documented (McConnell and Fenical, 1977; Woolard et al., 1979). In addition, the ultrastructure of the gland cells containing these localised metabolites and the mechanisms for their release into the environment is well understood (Paul et al., 2006a,b), as is the mechanism of action of these natural products against marine bacteria and invertebrate grazers (Paul et al., 2006b,c). This provides a platform from which to understand the fundamental biological and chemical mechanisms that deliver the anthelmintic properties observed in the present study.

Many genera of red algae are rich in natural anthelmintic products (Sato et al., 1996; Higa and Kuniyoshi, 2000). The best-known medicinal red seaweeds are *Digenea simplex* and *Chondria armata*, which have been utilised for centuries for the treatment of roundworms in humans in Asia and the active natural product in *D. simplex*, α-kainic acid, has been isolated and developed into an anthelmintic drug (Higa and Kuniyoshi, 2000). The extracts of *Ulva* sp. and *D. delicatula*, which also prolonged embryonation and reduced hatching success (Fig. 3), also contain natural products. *Ulva* is rich in dimethylsulfide and dimethylsulfoniopropionate (Karsten et al., 1991) while *Dictyopteris* contains halogenated sesquiterpenes and diterpenes (Song et al., 2005). Nevertheless, our results are unusual given that monogenean eggs have remarkable resistance to chemical treatments due to their sclerotised protein shell protecting the developing embryo (Kearn, 1986; Whittington and Chisholm, 2008).

Monogenean eggs are highly resistant to physical and chemical treatments and numerous chemicals (Aqui-S, benzalkonium chloride, caprylic acid, chlorine, copper, ethanol, formalin, sodium hypochlorite, sodium chloride, potassium permanganate, trichlorfon, methylene blue, praziquantel and zinc) have been investigated for their efficiency at preventing hatching of monogenean eggs with little success (Diggles et al., 1993; Yoshinaga et al., 2000; Sharp et al., 2004; Sitjà-Bobadilla et al., 2006). The most successful treatments are egg desiccation (Ernst et al., 2005; Chen et al., 2010) or immersion in water above 50 °C (Ernst et al., 2005), while hyposaline treatment (⩽15 ppt) remains the only method prohibiting *Neobenedenia* spp. from hatching entirely (Mueller et al., 1992; Ellis and Watanabe, 1993). However, hyposaline treatments are unsuitable on commercial scales due to osmotic stress for stenohaline fish species and increased host susceptibility to reinfection (Ohno et al., 2009). Hyposaline solutions also kill nitrifying bacteria in biological filtration systems commonly used in intensive finfish hatcheries.

Parasitic monogenean larval stages could be considered more susceptible to treatments compared with embryos protected by a proteinaceous egg shell and attached parasite stages that receive nutritional benefit from their host. Although high variance in oncomiracidia infection success negated statistically significant differences in this study, mean values were lower for extracts of *A. taxiformis* (51%) and *Ulva* sp. (54%) compared with the control (71%; Fig. 6). Extracts of *A. taxiformis* and *Ulva* sp. consistently reduced hatching success and/or delayed hatching, however the magnitudes of these effects were different in two experiments (see Figs. 3 and 5). This may be related to seasonal variation in natural products in seaweeds (Wright et al., 2000; Genovese et al., 2012) and/or differences in the source of parasites (two separate populations) and egg collection (in vitro versus in vivo) in the experiments. The natural extension of this work is to determine the efficacy of seaweed treatments in subsequent infection success of oncomiracidia that hatch from treated eggs.

In conclusion, natural treatments utilising water-soluble marine natural products extracted from seaweeds are simple to prepare and show potential for parasite management in aquaculture systems. Seaweed extracts can be applied directly in closed systems, while large scale aquaculture systems require integrated seaweed cultivation, which is already established in some finfish industries, primarily for bioremediation. Notably, there are no methods to prevent *Neobenedenia* spp. infections, and current parasite management efforts provide only short-term solutions by removing attached parasite stages (Whittington, 2012). However, *Neobenedenia* eggs are highly sensitive to the water-soluble extract of *A. taxiformis* and this treatment provides an effective mechanism to break the life cycle of this important ectoparasite.

Acknowledgements

We thank Richard Saunders, James Tuller, Thane Militz and Nico Neuveux for technical assistance, Ian Whittington for identifying the parasites, Leslie Chisholm for accessioning specimens to SAMA, and Good Fortune Bay Fisheries Ltd and Mainstream Aquaculture Ltd for providing fish and parasite specimens. We thank two anonymous reviewers for comments on the manuscript. This project was funded by a James Cook University (JCU), Australia, Faculty of Science and Engineering Grant, awarded to KSH. It was also supported by the Advanced Manufacturing Cooperative Research Centre (AMCRC) funded through the Australian Government's Cooperative Research Centre Scheme. This research is part of the MBD Energy Research and Development program for Biological Carbon Capture and Storage, Australia. Experiments were conducted under JCU Animal Ethics Approval (No. A1579).

References

Bondad-Reantaso, M.G., Ogawa, K., Fukudome, M., 1995. Reproduction and growth of *Neobenedenia girellae* (Monogenea: Capsalidae), a skin parasite of cultured marine fishes of Japan. Fish Pathol. 30, 227–231.

Blunt, J.W., Copp, B.R., Keyzers, R.A., Munro, M.H.G., Prinsep, M.R., 2012. Marine natural products. Nat. Prod. Rep. 29, 144–222.
Chambers, C.B., Ernst, I., 2005. Dispersal of the skin fluke *Benedenia seriolae* (Monogenea: Capsalidae) by tidal currents and implications for sea-cage farming of *Seriola* spp. Aquaculture 250, 60–69.
Chen, H.G., Chen, H.Y., Wang, C.S., Chen, S.N., Shih, H.H., 2010. Effects of various treatments on egg hatching of *Dendromonocotyle pipinna* (Monogenea: Monocotylidae) infecting the blotched fantail ray, *Taeniurops meyeni*, in Taiwan. Vet. Parasitol. 171, 229–237.
Chopin, T., Buschmann, A.H., Halling, C., Troell, M., Kautsky, N., Neori, A., Kraemer, G.P., Zertuche-Gonzalez, J.A., Yarish, C., Neefus, C., 2001. Integrating seaweeds into marine aquaculture systems: a key toward sustainability. J. Phycol. 37, 975–986.
Deveney, M.R., Chisholm, L.A., Whittington, I.D., 2001. First published record of the pathogenic monogenean parasite *Neobenedenia melleni* (Capsalidae) from Australia. Dis. Aquat. Organ. 46, 79–82.
Diggles, B.K., Roubal, F.R., Lester, R.J.G., 1993. The influence of formalin, benzocaine and hyposalinity on the fecundity and viability of *Polylabroides multispinosus* (Monogenea: Microcotylidae) parasitic on the gills of *Acanthopagrus australis* (Pisces: Sparidae). Int. J. Parasitol. 23, 877–884.
Dyer, W.G., Williams, E.H., Bunkley-Williams, L., 1992. *Neobenedenia pargueraensis* n. sp. (Monogenea: Capsalidae) from the red hind, *Epinephelus guttatus*, and comments about *Neobenedenia melleni*. J. Parasitol. 78, 399–401.
Ellis, E.P., Watanabe, W.O., 1993. The effects of hyposalinity on eggs, juveniles and adults of the marine monogenean, *Neobenedenia melleni*. Treatment of ecto-parasitosis in seawater-cultured tilapia. Aquaculture 117, 15–27.
Ernst, I., Whittington, I.D., Corneillie, S., Talbot, C., 2002. Monogenean parasites in sea-cage aquaculture. Austasia Aquaculture 22, 46–48.
Ernst, I., Whittington, I.D., Corneillie, S., Talbot, C., 2005. Effects of temperature, salinity, desiccation and chemical treatments on egg embryonation and hatching success of *Benedenia seriolae* (Monogenea: Capsalidae), a parasite of farmed *Seriola* spp. J. Fish Dis. 28, 157–164.
Fajer-Ávila, E.J., Velásquez-Medina, S.P., Betancourt-Lozano, M., 2007. Effectiveness of treatments against eggs, and adults of *Haliotrema* sp. and *Euryhaliotrema* sp. (Monogenea: Ancyrocephalinae) infecting red snapper, *Lutjanus guttatus*. Aquaculture 264, 66–72.
Genovese, G., Faggio, C., Gugliandolo, C., Torre, A., Spanò, A., Morabito, M., Maugeri, T.L., 2012. In vitro evaluation of antibacterial activity of *Asparagopsis taxiformis* from the Straits of Messina against pathogens relevant in aquaculture. Mar. Environ. Res. 73, 1–6.
Glennon, V., Chisholm, L.A., Whittington, I.D., 2006. Three unrelated species, 3 sites, same host–monogenean parasite of the southern fiddler ray, *Trygonorrhina fasciata*, in South Australia: egg hatching strategies and larval behaviour. Parasitology 133, 55–66.
Higa, T., Kuniyoshi, M., 2000. Toxins associated with medicinal and edible seaweeds. J. Toxicol.-Toxin Rev. 19, 119–137.
Hirayama, T., Kawano, F., Hirazawa, N., 2009. Effect of *Neobenedenia girellae* (Monogenea) infection on host amberjack *Seriola dumerili* (Carangidae). Aquaculture 288, 159–165.
Karsten, U., Wiencke, C., Kirst, G.O., 1991. Growth pattern and β-dimethylsulphoniopropionate (DMSP) content of green macroalgae at different irrandiances. Mar. Biol. 108, 151–155.
Kearn, G.C., 1986. The eggs of monogeneans. Adv. Parasitol. 25, 175–273.
Kearn, G.C., Ogawa, K., Maeno, Y., 1992. Hatching patterns of the monogenean parasites *Benedenia seriolae* and *Heteraxine heterocerca* from the skin and gills, respectively, of the same host fish, *Seriola quinqueradiata*. Zool. Sci. 9, 451–455.
Lio-Po, G.D., Leaño, E.M., Peñaranda, M.M.D., Villa-Franco, A.U., Sombito, D.C., Guanzon, N.G., 2005. Anti-luminous *Vibrio* factors associated with the 'green water' grow-out culture of the tiger shrimp *Penaeus monodon*. Aquaculture 250, 1–7.
Mata, L., Schuenhoff, A., Santos, R., 2010. A direct comparison of the performance of the seaweed biofilters, *Asparagopsis armata* and *Ulva* sp. J. Appl. Phycol. 22, 639–644.
McCloud, T.G., 2010. High throughput extraction of plant, marine and fungal specimens for preservation of biologically active molecules. Molecules 15, 4526–4563.
McConnell, O., Fenical, W., 1977. Halogen chemistry of the red alga *Asparagopsis*. Phytochemistry 16, 367–374.
Mueller, K.W., Watanabe, W.O., Head, W.D., 1992. Effect of salinity on hatching in *Neobenedenia melleni*, a monogenean ectoparasite of seawater-cultured tilapia. J. World Aquaculture. Soc. 23, 199–204.
Ogawa, K., Bondad-Reantaso, M.G., Fukudomw, M., Wakabayashi, H., 1995. *Neobenedenia girellae* (Hargis, 1955) Yamaguti, 1963 (Monogenea: Capsalidae) from cultured marine fishes of Japan. J. Parasitol. 81, 223–227.
Ogawa, K., Miyamoto, J., Wang, H.C., Lo, C.F., Kou, G.H., 2006. *Neobenedenia girellae* (Monogenea) infection of cultured cobia *Rachycentron canadum* in Taiwan. Fish Pathol. 41, 51–56.
Ohno, Y., Kawano, F., Hirazawa, N., 2009. The effect of oral antibiotic treatment and freshwater bath treatment on susceptibility to *Neobenedenia girellae* (Monogenea) infection of amberjack (*Seriola dumerili*) and yellowtail (*S. quinqueradiata*) hosts. Aquaculture 292, 248–251.
Paul, N.A., Cole, L., de Nys, R., Steinberg, P.D., 2006a. Ultrastructure of the gland cells of the red alga *Asparagopsis armata* (Bonnemaisoniaceae). J. Phycol. 42, 637–645.
Paul, N.A., de Nys, R., Steinberg, P.D., 2006b. Chemical defence against bacteria in the red alga *Asparagopsis armata*: linking structure with function. Mar. Ecol.-Prog. Ser. 306, 87–101.
Paul, N.A., de Nys, R., Steinberg, P.D., 2006c. Seaweed-herbivore interactions at a small scale: direct tests of feeding deterrence by filamentous algae. Mar. Ecol.-Prog. Ser. 323, 1–9.
Rückert, S., Palm, H.W., Klimpel, S., 2008. Parasite fauna of seabass (*Lates calcarifer*) under mariculture conditions in Lampung Bay. Indones. J. Appl. Ichthyol. 24, 321–327.
Sato, M., Naakano, T., Taeuchi, M., Kanno, N., Nagahisa, E., Sato, Y., 1996. Distribution of neuroexcitatory amino acids in marine algae. Phytochemistry 42, 1595–1597.
Selvin, J., Manilal, A., Sujith, S., Kiran, G.S., Liption, A.P., 2011. Efficacy of marine green alga *Ulva fasciata* extract on the management of shrimp bacterial diseases. Latin Am. J. Aquat. Res. 39, 197–204.
Sharp, N., Diggles, B.K., Poortenaar, C.W., Willis, T.J., 2004. Efficacy of Aqui-S, formalin and praziquantel against the monogeneans, *Benedenia seriolae* and *Zeuxapta seriolae*, infecting yellowtail kingfish *Seriola lalandi lalandi* in New Zealand. Aquaculture 236, 67–83.
Sitjià-Bobadilla, A., Conde de Felipe, M., Alvarez-Pellitero, P., 2006. *In vivo* and *in vitro* treatments against *Sparicotyle chrysophrii* (Monogenea: Microcotylidae) parasitizing the gills of gilthead sea bream (*Sparus aurata* L.). Aquaculture 261, 856–864.
Smit, A.J., 2004. Medicinal and pharmaceutical uses of seaweed natural products: a review. J. Appl. Phycol. 16, 245–262.
Song, F.-H., Fan, X., Xu, X.-L., Zhao, J.-L., Han, L.-J., Shi, J.-G., 2005. Chemical constituents of the brown alga *Dictyopteris divaricata*. J. Asian Nat. Prod. Res. 7, 777–781.
Sudheer, N.S., Philip, R., Singh, I.S.B., 2011. *In vivo* screening of mangrove plants for anti WSSV activity in *Penaeus monodon*, and evaluation of *Ceriops tagal* as a potential source of antiviral molecules. Aquaculture 311, 36–41.
Thoney, D.A., Hargis Jr., W.J., 1991. Monogenea (Platyhelminthes) as hazards for fish in confinement. Ann. Rev. Fish Dis. 1, 133–153.
Troell, M., 2009. Integrated marine and brackishwater aquaculture in tropical regions: research, implementation and prospects. In: Soto, D. (Ed.), Integrated mariculture: a global review. FAO Fisheries and Aquaculture Technical Paper, Rome, pp. 47–131.
Whittington, I.D., 2004. The Capsalidae (Monogenea: Monopisthocotylea): a review of diversity, classification and phylogeny with a note about species complexes. Folia Parasitol. 51, 109–122.
Whittington, I.D., Chisholm, L.A., 2008. Diseases caused by Monogenea. In: Eiras, J.C., Segner, H., Wahlii, T., Kapoor, B.G. (Eds.), Fish Diseases Volume 2. Science Publishers Inc., Enfield, pp. 683–816.
Whittington, I.D., Horton, M.A., 1996. A revision of *Neobenedenia* Yamaguti, 1963 (Monogenea: Capsalidae) including a redescription of *N. melleni* (MacCallum, 1927) Yamaguti, 1963. J. Nat. Hist. 30, 1113–1156.
Whittington, I.D., 2012. *Benedenia seriolae* and *Neobenedenia* Species. In: Woo, P.T.K., Buchmann, K. (Eds.), Fish Parasites Pathobiology and Protection. CAB International, Oxfordshire, UK, pp. 225–244.
Williams, R.E., Ernst, I., Chambers, C.B., Whittington, I.D., 2007. Efficacy of orally administered praziquantel against *Zeuxapta seriolae* and *Benedenia seriolae* (Monogenea) in yellowtail kingfish *Seriola lalandi*. Dis. Aquat. Organ. 77, 199–205.
Woolard, F.X., Moore, R.E., Roller, P.P., 1979. Halogenated acetic and acrylic acids from the red alga *Asparagopsis taxiformis*. Phytochemistry 18, 617–620.
Wright, J.T., de Nys, R., Steinberg, P.D., 2000. Geographic variation in halogenated furanones from the red alga *Delisea pulchra* and associated herbivores and epiphytes. Mar. Ecol.-Prog. Ser. 207, 227–241.
Yoshinaga, T., Segwa, I., Kamaishi, T., Sorimachi, M., 2000. Effects of temperature, salinity, and chlorine treatment on egg hatching of the monogenean *Neoheterobothrium hirame* infecting Japanese flounder. Fish Pathol. 35, 85–88.