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Combined effects of UV irradiation, ozonation, and the probiotic *Bacillus* spp. on growth, survival, and general fitness in European lobster (*Homarus gammarus*)

Karen L. Middlemiss [a,*], Carly L. Daniels [b], Mauricio A. Urbina [a], Rod W. Wilson [a,*]

[a] Biosciences, College of Life and Environmental Sciences, Geoffrey Pope Building, University of Exeter, Stocker Road, Exeter, EX4 4QD, UK
[b] The National Lobster Hatchery, South Quay, Padstow, Cornwall PL28 8BL, UK

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ABSTRACT

Bacterial pathogens are a leading cause of disease in hatchery aquaculture systems and preventative methods such as use of probiotics as feed supplements and water additives are well documented. However, comparisons between the effectiveness of using probiotic water additives over traditional biocontrol methods are less understood. This study assessed the combined effects of ultraviolet (UV) irradiation, O_3 (ozone) and *Bacillus* spp. as a water additive (probiotic), in the culture of European lobster (*Homarus gammarus*) in a semi-closed recirculation system. Larvae were categorised as zoea stages I–III, megalopa (stage IV) and juvenile (stage V) onwards. Stage I larvae were assigned to one of six treatment groups consisting of 1) O_3, 2) probiotic, 3) probiotic + O_3, 4) probiotic + O_3+ UV, 5) O_3 + UV, or 6) probiotic + UV, for 18 days. During stages I–V, growth was measured on 1, 6, 11, 18, 24, and 31 dph (days post hatch), and survival was measured on 1, 18, 24 and 31 dph. Bacterial counts of pathogenic *Vibrio* spp. in culture water were measured at 1, 4, 9, 14, and 18 dph. Lobsters were also exposed to a physiological fitness test (low salinity challenge) at stage IV, 7 days post treatment. Results showed that O_3 is comparatively more beneficial than probiotic with increased LWG (live weight gain) in the O_3 treatment over probiotic between stage IV and V (>5 mg). Survival rates were ~10% higher in the O_3 treatment group than probiotic treatment group on day 18, then ~5% and ~4% higher on days 24 and 31. Lobster biomass on day 18 was ~60% higher in the O_3 treatment than probiotic treatment and 116% higher on day 31. Total *Vibrio* spp. present in the O_3 treatment was 0.05% of the total in the probiotic treated culture water (day 18). Results between UV treatment groups showed significantly lower numbers of *Vibrio* spp. present in probiotic + O_3 + UV culture water 4 dph than O_3 + UV (~10 fold higher) or UV + probiotic (~15 fold higher) and by day 18 probiotic + O_3 + UV was significantly higher than O_3 + UV (~8 fold higher). Osmoregulatory challenge test resulted in no significant differences in physiological fitness between any treatment groups. The present study shows the effectiveness of O_3 in aquaculture facilities for control of pathogens in the rearing of European lobster over either a probiotic water additive (at 3.75×10^7 CFUs L^{-1}) or UV irradiation.

1. Introduction

Due to the explosive growth of human population and consequent increase in the demand for food, aquaculture and fisheries are one of the fastest growing industries (Food and Agriculture Organization of the United Nations, FAO, 2009). Crustaceans are a large part of the seafood market and demand has increased steadily. King crabs (Urbina et al., 2013), penaeid shrimps and lobsters are probably the most economically valued decapod crustacean species (Food and Agriculture Organization of the United Nations, FAO, 2014). The European lobster, *Homarus gammarus* (L.), belongs to the order Decapoda, and their natural habitat encompasses a large latitudinal and temperature range. Their habitat extends predominantly along the Eastern Atlantic Ocean, from the cool waters of Norway to the more temperate waters of the Morocco and Mediterranean coastline (Triantafyllidis et al., 2005). The European lobster is usually found in depths up to 50 m, but it has been reported to inhabit down to 150 m (Cobb and Castro, 2006). The European lobster is commercially fished throughout its distribution and annual global catches have shown a steady increase since 1950 (Food and Agriculture Organization of the United Nations, FAO, 2014). In the early 2000s total annual landings for lobsters in the UK were around 1300 T and 10 years later this figure had more than doubled to around 3000 T (Centre for Environment, Fisheries and Aquaculture Science, CEFAS, 2014). With landings valued at around 31 million pounds sterling, lobsters constitute around 40% of the value of all annual UK shellfish landings. As with many marine species, intensive harvesting of the European lobster historically

* Corresponding authors. Tel.: +44 1392 725171; fax: +44 1392 263434.
E-mail addresses: kombi@xtra.co.nz (K.L. Middlemiss), r.w.wilson@exeter.ac.uk (R.W. Wilson).

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contributed to a general population decline. This resulted in UK legislative and conservation measures which include enhancement of wild stock populations through hatchery based culture of larvae for release into coastal marine waters (Agnalt et al., 2004; Bannister and Addison, 1998; Tully, 2004; van der Meeren, 2005).

Bacterial pathogens, frequently found in seawater and food sources, are a leading cause of disease-related mortality common to aquaculture facilities (Goulden et al., 2012; Jithendran et al., 2010; Silva et al., 2013). It has even been suggested that the intensive nature of larviculture has selected for virulent strains of the gram negative genus *Vibrio* which are pathogenic to decapod larvae (Goulden et al., 2012) causing reduced growth and survival (Bourne et al., 2007). Major routes of infection in aquatic species include the gills and gastrointestinal tract (Goulden et al., 2012). Animals are particularly vulnerable after a moult (especially larvae because of the high frequency of moults) when the new exoskeleton is thinner and not yet hardened (Cawthorn, 1997). However, infection from injury is also likely to occur due to their highly cannibalistic behaviour (Scolding et al., 2012). Traditional antimicrobial control methods in aquaculture including the use of antibiotics and chemical treatment of water using UV irradiation and O_3 (Brown and Russo, 1979; Scolding et al., 2012) are being challenged by recent research on the alternative use of probiotics to improve water quality and for pathogen control (Moriarty, 1998). Probiotics are described as microbial supplements that confer health benefits through modulation of bacterial communities (Gatesoupe, 1999).

It has been found that higher doses of UV effectively removed about 98% of heterotrophic bacteria in salmon farming on a recirculating system (Sharrer and Summerfelt, 2007a). Combined use of UV and O_3 resulted in almost total elimination of bacteria in an Arctic Char freshwater recirculation system (Sharrer and Summerfelt, 2007b). Ozone also effectively eliminates bacterial and viral pathogens on both the host and in the water (Emerson et al., 1982; Scolding et al., 2012; Sellars et al., 2005). Whilst O_3 has been considered to be more effective than UV in some cases (Liltved et al., 1995), it has also been reported that its application could negatively affect host species by causing tissue damage (Ritola et al., 2002). The normal use of O_3 is in application to the water treatment system, with residuals then removed before contact with animals. Both O_3 and UV are clearly efficient at controlling bacterial pathogens, but their real advantages for farming species are not yet clear. The use of both O_3 and UV as an antimicrobial treatment has been effective at controlling bacterial pathogens without harming the host in studies on European lobster (Daniels et al., 2010; Scolding et al., 2012). However, synergies between direct application of probiotics to culture water in combination with both O_3 and UV have not been evaluated in semi-closed recirculating systems.

In recent years growth and survival of many aquatic species, including crustaceans, have significantly improved through the alternative, and well accepted use of probiotics, including the genus *Bacillus*, as both a feed supplement and water additive (e.g. Daniels et al., 2010, 2013; de Souza et al., 2012; Hai et al., 2009; Zhang et al., 2011). Probiotics confer health benefits through various mechanisms including 1) competitive exclusion of pathogenic bacteria (e.g. *Vibrio* spp.) from adhesion sites in the gills and gastrointestinal tract, 2) production of inhibitory compounds, 3) improvement of digestive enzymatic activity, 4) nutrient provision, 5) immunostimulants, and 6) improved water quality (Balcazar et al., 2006; Cha et al., 2013; Gullian et al., 2004; Irianto and Austin, 2002; Merrifield et al., 2010). In aquatic species, *Vibrio* spp. present the biggest pathogenic threat as they grow quickly and adapt to changing environmental conditions (Battison et al., 2008; de Souza et al., 2012; Maeda et al., 1997). *Bacillus* spp. are commercially available gram-positive spore forming bacteria used as probiotics and have been shown to benefit European lobster when administered as a feed supplement (Daniels et al., 2010). Positive results have also been found when used as a water additive in shrimp species (Cha et al., 2013). Dietary probiotics have been used to improve growth and survival of European lobster (Daniels, 2011; Daniels et al., 2010, 2013). There are cost benefits to be made from administering probiotics as a water additive to both improve water quality and provide biocontrol over the traditional and more expensive methods of UV and O_3.

This study aims to compare the single and synergistic effects of *Bacillus* spp. administered as a water additive alone and with UV and O_3 to control pathogens in the culture of European lobster. It was hypothesised that *Bacillus* spp. (referred to herein as 'probiotic') would confer supplementary health benefits to the host in addition to traditional biocontrol and water quality methods.

2. Materials and methods

2.1. Experimental animals

Experiments were undertaken at the National Lobster Hatchery (NLH) larval rearing research facility in Padstow, North Cornwall, UK, during October and December 2013, just after the natural breeding season (April–September). Water for the aquaria was pumped from the surrounding Camel Estuary (Padstow, UK, −50° 32′ 19.67″ N, 4° 56′ 5.85″ W) at high tide into a reservoir and treated using glass artificial filter media providing mechanical filtration to 50 μM in addition to UV irradiation before use in aquaria. Ovigerous adult female European lobsters were collected from various locations along the Cornish coast and held in 6 °C cold water storage tanks at the NLH until required. Lobsters were gradually acclimated over a period of ~6 days to 19 °C and placed in a recirculating and aerated broodstock tank at a salinity of 35 PSU and sustained with a diet of blue mussels (*Mytilus edulis*). Larvae hatched overnight from several females (different broodstock were used for each trial and there was a 5 week difference in trial start dates) creating a pool of larvae which were then treated in a Chloramine-T (Pharmaq, UK) bath (0.04 g L^{-1}) for 1 h before being transferred to experimental aquaria. Larval stages were classified according to the conventional and pre-established developmental stages. Stages I–V were used in this study, whereby stages I–III are zoea, stage IV is a megalopa and stage V is a post larvae (PL) or juvenile (Factor, 1995).

2.2. Rearing systems

2.2.1. Experimental design

It should be noted that due to animal welfare concerns the NLH could not allow the use of a 'control' in the form of completely untreated seawater and therefore, due to facility restrictions, this was not included as part of the experimental design. All seawater coming into the NLH from the nearby estuary is treated via mechanical filtration and UV irradiation and held in a reservoir prior to use in experiments. Therefore the O_3 (Trial 1) and O_3 + UV (Trial 2) treatment groups were defined as the controls for each of the trials respectively (see below). Previous studies conducted at the NLH on benefits of O_3 water treatments by Scolding et al. (2012) were conducted similarly with the 'control' being UV treated seawater with no O_3.

Experimental aquaria consisted of three separate semi-closed recirculating systems each supporting 4 replicate 80 L up-welling Kreisel cones (i.e. 12 cones in total) maintained at a water flow rate of ~1800 L h^{-1} per system. Each system was randomly assigned to each treatment (thus providing four replicates per treatment). Aeration of cones was maintained to a level which provided dissolved oxygen in the range of 8.1–8.9 mg L^{-1}, and active water mixing as a strategy to limit conspecific contact. Each system contained approximately 600 L of seawater and the setup consisted of: filter sock, protein skimmer, bio-filter, sand filter and ozonation via the protein skimmer and or irradiated by 2 × 55-W UV steriliser where treatment required. Water tests were carried out every second day to measure total ammonia, NO_2^-, NO_3^-, salinity, pH, temperature and dissolved oxygen (DO) in all systems. A saltwater master test kit (API® Mars Inc.), H_2 ocean salinity refractometer, HQ11d pH and temperature meter (Hatch, Salford, UK) and DO probe (OxyGuard Handy-Alpha, Sterner AquaTech, UK) were