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Evidence-based practice

The effect of Virkon® Aquatic on the bacterial count of *Artemia* cultures

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Abstract

The use of Artemia as a live food in larval rearing is widespread; however several studies have demonstrated that *Artemia* nauplii are a vector for introducing potentially harmful bacteria. A variety of methods have been investigated to reduce the bacterial contamination in *Artemia* including antibiotics, probiotics, ozone and chemical treatments. Virkon® Aquatic is a broad spectrum disinfectant widely used in the aquarium industry; this research looked at the potential of it to reduce bacterial loading of *Artemia* nauplii. Newly hatched *Artemia* were placed in a Virkon® Aquatic dip at six different dosage rates (10, 20, 40, 80, 100 and 120 ppm) for 1 hour, with a 0 ppm control. Compared to the control samples, exposure to 10 ppm and 120 ppm Virkon® Aquatic resulted in a 93.76% and 99.78% decrease in bacteria loading, respectively. Therefore, the results show that the novel use of this disinfectant in the reduction of bacterial loads on *Artemia* is highly effective, and has practical applications for both aquaculture and the aquarium industry.

Background

Despite considerable progress in formulated larval feeds and feeding techniques, the worldwide use of live feeds is expected to remain essential in the future (Høj et al. 2009; Dhont and Dierckens 2013; FAO 2015). *Artemia* are a very popular live food across the aquaculture and aquarium industry due to their ease of culture and quick hatch rate; and are most commonly used as freshly hatched nauplii (Barnabe 1990; Hendry et al. 2001; Lavens and Sorgeloos 2000; Dhont and Dierckens 2013). It has been estimated that *Artemia* nauplii are fed to over 85% of aquaculture species around the world (Hameed and Balasubramanian 2000) and most cultured ornamental species (Olivotto et al. 2008).

Despite their widespread application, the bacterial load associated with *Artemia* naulpii remains a major concern with their use (Agh et al. 2001; Tolomei et al. 2004; Hoj et al. 2009; Dhont and Dierckens 2013; Hache et al. 2016). Several studies demonstrate that *Artemia* nauplii are a vector for introducing potentially harmful bacteria to larvae with reported loads up to 10^8 colony forming units (CFU) per ml of *Artemia* homogenate (Agh et al. 2001; Dhont and Dierckens 2013; Hameed and Balasubramanian 2000; Høj et al. 2009; Interaminense et al. 2014). Moreover, many studies have linked the feeding of *Artemia* with bacterial infections and mortalities in popular aquarium species such as seahorses (*Hippocampus spp.*) (Wilson and Vincent 2000; Lin et al. 2016; Wang et al. 2016) and clownfish (*Amphiprion*) species (Siva et al. 2014).

Dry *Artemia* cysts have a very low number of associated bacteria, but the culture water when cysts hatch is rapidly colonised by bacterial species (Sorgeloos et al. 2001; Pintado et al. 2014). Glycerol is released at hydration of the cysts and offers an ideal culture medium for opportunistic bacteria, such as *Vibrio* species, which may be a threat to the health of the larvae feeding on the *Artemia* (Agh et al. 2001; Sorgeloos et al. 2001; Høj et al. 2009; Pintado et al. 2014). A number of

Table 1. Comparative effectiveness of different *Artemia* disinfection methods.

Method	Effect	Reference
Formalin	96.1% reduction in bacteria numbers compared to control	Gomez-Gil et al. 1994
Sanocare (24 h treatment)	53.3% reduction in bacteria numbers compared to control	Mzimba and Steinarsson 2014
Pyceze (24 h treatment)	84% reduction in bacteria numbers compared to control	Mzimba and Steinarsson 2014
Antibiotics	Improved turbot larvae survival (fed Artemia) by 337.0%	Perez and Gatesoupe 1988
	93.1% reduction of bacteria on decapsulated Artemia cysts	Interaminense et al. 2014
Probiotics	95.3% reduction on decapsulated Artemia cysts	Interaminense et al. 2014
Ozone (0.75 g L^{-1})	>99.9% reduction in bacteria numbers compared to control	Theisen et al. 1998

methods for disinfecting *Artemia* have been investigated, including antimicrobials (Table 1). However, concerns have been expressed regarding the efficacy, reliability and safety of the methods, as well as the potential encouragement of antibiotic resistant bacteria strains (Hameed and Balasubramanian 2000; Tolomei et al. 2004; Andrews et al. 2011; Hache et al. 2016).

Virkon® Aquatic is a potassium monopersulphonate triple salt-based disinfectant that has been marketed as the most proven livestock disinfectant in the world (AASCO 2016). Contact time is very quick, taking 5–10 minutes (Chemours 2015; AASCO 2016). In an independent trial, it was reported to kill up to 99.99% of a wide range of aquatic pathogens including 31 bacterial strains, 58 viruses and six fungi species with no evidence of resistance (Mzimba and Steinarsson 2014). Furthermore, it has a very low toxicity to animals (Schmidt et al. 2009). It is one of the very few US Environmental Protection Agency registered disinfectants labelled specifically for use in fish culture and is also registered under European guidelines (1907/2006). Though primarily used within aquaculture and aquarium industry for the disinfection of equipment (Dvorak 2009), Virkon® has been used experimentally in the disinfection of *Artemia*, but only in combination with antibiotics (Høj et al. 2009). Here we exposed *Artemia* nauplii with different concentrations of Virkon® (0–120 ppm) without the addition of antibiotics and observed the impact on total bacterial levels.

Action

Test organism

Cysts of *Artemia fransiscana* (ZM Systems, Winchester, UK) were hatched under optimum conditions (temperate 28°C, salinity 35 g/l, pH 8–8.3, illumination 2,000 lux and cyst density of 1.0 g/l; oxygen >4 mg/l; Holt 2011; Southgate 2012; FAO 2015) at the Aquatic Research and Conservation Centre, University Centre Sparsholt. Cysts were hatched in hatching cones within a water bath to maintain consistent temperature and vigorously aerated to keep the cysts in suspension. Triplicate cones were used for every concentration of treatment. Hatching took 24 h after which Virkon® Aquatic concentrations were added at six different dose rates with a control. A 1000-ppm stock solution was prepared by dissolving 1 g of Virkon® Aquatic per litre of 35 g/l saltwater. Then, volumes of 0, 10, 20, 40, 80, 100 and 120 ml were dispensed into each *Artemia* culture to achieve final concentrations of 0 (control), 10, 20, 40, 80, 100 and 120 ppm.

Experimental design

Range finding trials (unpublished) were used to establish an appropriate series of test concentrations. After dosing the cultures, the *Artemia* were left for a further hour in the Virkon® solution under continual aeration (from the base of the vessel) to maintain suspension of the nauplii. *Artemia* culture samples (20 ml) were then pipetted into sterile 100 ml glass bottles for dilution and bacterial counts. Culture samples were diluted with sterile 35 g/l saline water. Samples were diluted to 1:10 and 1:1000 for the Virkon® treated cultures, and 1:1000 for control samples. After dilution, 0.1 ml of sample was plated onto 90 ml Tryptone soya agar (TSA) (Oxoid, Basingstoke, UK) plates. Every dilution was triplicated. The plates were cultured for 36 hours at 20°C. To evaluate the nauplii hatch rate, *Artemia* culture sample bottles were inverted to equally distribute the nauplii before 0.1 ml was pipetted onto an empty 90 ml Petri dish and counted under a stereomicroscope. Three separate counts were taken for each replicate.

Statistical analyses

Normal distribution of data and homoscedasity were tested using the Shapiro-Wilk test and Levene's test, respectively (Sokal and Rohif 2012). Where the assumptions of a parametric test were met, a one-way ANOVA analysis was used to assess for significant differences in the means of the bacterial counts and hatch rates between treatments. Where the assumptions were not met, a Welch's ANOVA was used which does not assume equal variance. Where significant differences occurred in the ANOVA analyses (P=0.05), a post hoc Tukey's Honest Significant Difference test was applied to determine which means were significantly different.

Consequences

Disinfection

Data represents means of triplicate analyses. All Virkon® treatments resulted in significant reduction in bacterial levels (P=0.005) of between 91.81–99.78% compared to controls, without any significant differences between the treatments (Table 2).

Table 2. Bacterial colony counts (CFU) of Artemia culture water after incubation at 20°C on TSA (per 0.1 ml). All counts represent the mean (±S.E.) (n=9).

Treatment (ppm Virkon® Aquatic)	Colony count	Percentage of bacterial load reduction
0 (control)	2.01×10^6 (2.8×105)[a]	
10	1.3×10^5 (5.7×104)[b]	93.764
20	1.6×10^5 (3.7×104)[b]	91.807
40	3.4×10^4 (1.8×104)[b]	98.308
80	7.5×10^3 (5.9×103)[b]	99.628
100	4.7×10^3 (4.1×103)[b]	99.767
120	4.4×10^3 ($1.9 \times$x 103)[b]	99.781

Hatch rate

The hatch rate data showed there was no significant difference between any of the treatments and control (P=0.545) (Table 3). There were no mortalities or negative impacts on population density observed.

Discussion

The bacterial load in the control treatments were typical of those found in a commercial *Artemia* hatchery (e.g. 10^6–10^7 mL^{-1}; Dehasque et al. 1991). By applying even the lowest dose (10 ppm) of Virkon® Aquatic there was reduction of 93.76% in bacterial loading. Furthermore, from the control to the strongest dose (120 ppm), there was a bacterial decrease of 99.78%. Moreover, the treatment achieved this level of bacterial reduction without any significant impact on the hatch rate and initial survival of the *Artemia* nauplii, though further research is required on longer term effects.

Table 3. Hatch rate (*Artemia* nauplii per 0.1 ml) after 1 h treatment with Virkon® Aquatic. All counts represent the mean (S.E.) (n=9).

Treatment (ppm Virkon® Aquatic)	Number of nauplii
0 (control)	36.7 (3.4)
10	29.5 (2.1)
20	34.3 (2.4)
40	27.8 (3.1)
80	29.8 (2.7)
100	31.3 (0.6)
120	31.5 (1.9)

Disinfection methods vary in their efficacy and toxicity. In the current study, when bacterial reduction and nauplii survival are both considered, Virkon® had a superior effect over the comparative chemical methods of disinfection. Though higher eradication rates can be associated with the use of ozone, there are very clear toxicity issues to humans, fish and invertebrates (Andrews et al. 2011). Another disinfectant associated with high reduction in bacterial load was antibiotics. However, studies have demonstrated an increase in prevalence of antibiotic resistant bacteria – up to 60% – when antibiotics are used as a disinfectant (Hameed and Balasubramanian 2000) resulting in very variable efficacy. Høj et al. (2009) reported a substantial reduction in bacterial loads of *Artemia* after treatment with a combination of formalin, Virkon® S (reformulated as Virkon® Aquatic) and a mixture of antibiotics. However, the bacteria that survived included pathogenic genera such as *Vibrio*, which not only negates the fundamental point of disinfection, but could potentially facilitate the dominance of resistant pathogenic bacteria.

Not only does any treatment need to be non-toxic to the nauplii, but also to the organism to which it is fed. Virkon® has minimal environmental impact (Dunowska et al. 2005; AASCO 2016) and the risks to fish are low (Stockton-Fiti and Moffitt 2017). Any external residues can be washed off the nauplii by thoroughly rinsing with freshwater prior to feeding. The Virkon® will not have been ingested by the *Artemia*; nauplii do not feed exogenously until they enter instar II stage (8–10 hours post hatch) (Dhont and Van Stappen 2003; Tomkins and Dann 2009; Holt 2011; Southgate 2012). Field trials are currently ongoing in commercial cyprinid farms with the use of Virkon® Aquatic as an *Artemia* disinfectant with promising initial results (Davis personal communication; Haughton personal communication).

FAO (2015) state that one of the main issues with *Artemia* usage is microbiological contamination, with live feeds inputting pathogens and high bacterial loads to larvae cultures. Therefore, the application of a novel method of disinfection, which is not only highly effective, but also cost efficient, is likely to prove highly advantageous for marine hatcheries, public aquaria and ornamental hobbyists. Benefits could include greater survival of marine larvae, reduced risk of pathogen contamination of culture areas and improved fish welfare.

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References

AASCO - Advance Animal Science Company Ltd. (2016) The ultimate broad spectrum virucidal disinfectant – Virkon® Aquatic. Accessed February 15, 2016. Retrieved from: http://advance-animalbd.com/imgs/RuminantANDCompanionAnimal/Disinfectants/VIRKON-S.pdf

Agh N., Noori F., Asefi A., Sorgeloos P. (2001) *Effects of antibacterial agents on the hatch percentage and bacterial load in the hatching medium of* Artemia urmina *cysts.* In Hendry C.I., Van Stappen G., Wille M., Soregeloos ??. (Eds) Larvi 2001. Belgium: European Aquaculture Society.

Andrews C., Exell A., Carrington N. (2011) *The manual of fish health (Revised)*. Surrey: Interpet Publishing.

Barnabe G. (1990) *Aquaculture Volumes 1 and 2*. Great Britain: Ellis Horwood.

Chemours (2015) Virkon S tablets. Accessed May 18, 2015. Available at: https://www.chemours.com/Disinfectants_EMEA/en_GB/products/disinfectants/virkon_s_tablets.html

Dehasque M., Verdonck L., Sorgeloos P., Swings J., Léger P., Kersters K. (1991) Determination of the bacterial contamination in live food production systems in marine fish hatcheries in southern Europe. *Larvi* 91: 399–402.

Dhont J., Dierckens K. (2013) Rotifers, *Artemia* and copepods as live feeds for fish larvae in aquaculture. In: Allan G., Burnell G. *Advances in aquaculture hatchery technology.* Cambridge: Woodhead Publishing Limited.

Dunoswka M., Morley P.S., Hyatt D.R. (2005) The effect of Virkon® fogging on survival of *Salmonella enterica* and *Staphylococcus aureus* on surfaces in a veterinary teaching hospital. *Veterinary Microbiology 105*: 281–289.

Dvorak G. (2009) *Biosecurity for aquaculture facilities in the North Central Region. Iowa State University Digital Repository*. Retrieved from: https://lib.dr.iastate.edu/cgi/viewcontent.cgi?referer=http://scholar. FAO (2015) Cultured Aquatic Species Information Programme - *Artemia*. Retrieved from: http://www.fao.org/fishery/culturedspecies

Gimenez G., Padros F., Roque A. Estevez A., Furones D. (2006) Bacterial load reduction of live prey for fish larval feeding using Ox-Aquaculture. *Aquaculture Research* 37: 1133–1139.

Gomez-Gil R.S.B., Abreu-Grobois F.A., Romero-Jarero J., De Los Herrera-Vega M. (1994) Chemical disinfection of *Artemia* nauplii. *Journal of the World Aquaculture Society* 25(4): 579–583. https://doi.org/10.1111/j.1749-7345.1994.tb00829.x

Hache R., Lanteigne C., Hebert Y. (2016) Salt as a decontamination agent to control bacterial load in *Artemia* salina cultures. *Aquaculture* 452: 24–27. https://doi.org/10.1016/j.aquaculture.2015.10.017

Hameed A.S., Balasubramanian G. (2000) Antibiotic resistance in bacteria isolated from *Artemia* nauplii and efficacy of formaldehyde to control bacterial load. *Aquaculture* 183: 195–205. https://doi.org/10.1016/S0044-8486(99)00293-8

Hendry C.I., Van Stappen G., Wille M., Sorgeloos, ??. (2001). *Larvi* 2001. Belgium: European Aquaculture Society.

Høj L., Bourne D.G., Hall M.R. (2009) Localization, abundance and community structure of bacteria associated with *Artemia*: Effects of nauplii enrichment and antimicrobial treatment. *Aquaculture* 293(3–4): 278–285.

Holt G.J. (2011) *Larval fish nutrition*. Chichester: John Wiley & Sons.

Interaminense J.A., Ferreira Calazans N., do Valle B.C., Lyra Vogeley J., Peixoto S., Soares R., Lima Filho J.V. (2014) *Vibrio* spp. Control at Brine Shrimp, *Artemia*, Hatching and Enrichment. *Journal of the World Aquaculture Society* 45: 65–74. https://doi.org/10.1111/jwas.12096

Lavens P., Sorgeloos P. (2000) The history, present status and prospects of the availability of *Artemia* cysts for aquaculture. *Aquaculture* 181: 397–403. https://doi.org/10.1016/S0044-8486(99)00233-1.

Lin T., Zhang D., Liu X., Xiao D. (2016) Variations of immune parameters in the lined seahorse *Hippocampus erectus* after infection with enteritis pathogen of *Vibrio parahaemolyticus*. *Fish & Shellfish Immunology* 50: 247–254.

Liltved H., Hektoen H., Efraimsen H. (1995) Inactivation of bacterial and viral fish pathogens by ozonation or UV irradiation in water of different salinity. *Aquacultural Engineering* 14: 107–122. Mzimba L.M., Steinarsson A. (2014) The effect of disinfection on survival and feed quality of rotifers (*Arachionus plicatilis*) and brine shrimp (*Artemia salina*) United Nations University Fisheries Training Programme, Iceland. http://www.unuftp.is/static/fellows/document/lucia13prf.pdf

Olivotto I., Buttino I., Borroni M., Piccinetti C.C., Malzone M.G., Carnevali O. (2008) The use of the Mediterranean calanoid copepod Centropages typicus in Yellowtail clownfish (*Amphiprion clarkii*) larviculture. *Aquaculture* 284: 211–216.

Perez G., Gatesoupe F. (1988) Bacteria associated with cultured rotifers and *Artemia* are detrimental to larval turbot, *Scophthalmus maximus* L. *Aquacultural Engineering* 7: 289–293. https://doi.org/10.1016/0144-8609(88)90028-3.

Pintado J., Planas M., Makridis P. (2014) Live feeds: microbial assemblages, probiotics and prebiotics. In Merrifield, D., Ringo, E. *Aquaculture nutrition: Gut health, probiotics and prebiotics*. Chichester: John Wiley & sons.

Schmidt B.R., Geiser C., Peyer N., Keller N., von Rütte M. (2009) Assessing whether disinfectants against the fungus *Batrachochytrium dendrobatidis* have negative effects on tadpoles and zooplankton. *Amphibia-Reptilia* 30(3): 313–319. https://doi.org/10.1163/156853809788795245.

Siva M.U., Marudhupandi T., Haq M.A.B., Thankappan T. (2014) Histopathological study of lymphocystis disease virus (LCDV) in cultured false clownfish, *Amphiprion ocellaris* (Cuvier, 1830) and true clownfish. *Journal of Coastal Life Medicine* 2(4): 264–269.

Sokal R.R., Rohlf F.J. (2012) *Biometry* (4th edn). WH Freeman and Company. San Francisco.

Sorgeloos P., Dhert P., Candreva P. (2001) Use of the brine shrimp, *Artemia spp.,* in marine fish larviculture. *Aquaculture* 200: 147–159. https://doi.org/10.1016/S0044-8486(01)00698-6.

Southgate P.C. (2012) Foods and Feeding. In Lucas, J, S., Southgate, P, C. *Aquaculture farming aquatic animals and plants*. (2nd ED). Chichester: Wiley-Blackwell.

Stockton-Fiti K.A., Moffitt C.M. (2017) Safety and efficacy of Virkon® aquatic as a control tool for invasive Molluscs in aquaculture. *Aquaculture* 480: 71–76. https://doi.org/10.1016/j.aquaculture.2017.08.005.

Sugita H., Asai T., Hayashi K., Mitsuya T., Amanuma K., Maruyama C., Deguchi Y. (1992) Application of ozone disinfection to remove *Enterococcus seriolicida, Pasteurella piscicida*, and *Vibrio anguillarum* from seawater. 58: 4072–7075. https://doi.org/10.1163/S0044-8486(01)00698-6

Theisen D.D., Stansell D.D., Woods C. (1998) Disinfection of Nauplii of *Artemia* by Ozonation. *The progressive fish-culturist*. 60: 149–151. https://doi.org/10.1577/1548-8640(1998)060<0149:DONOAF>2.0.CO;2.

Tolomei A., Burke C., Crear B., Carson J. (2004) Bacterial decontamination of on-grown *Artemia*. *Aquaculture*. 232: 357–371. https://doi.org/10.1016/S0044-8486(03)00540-4.

Wang X., Zhang Y., Qin G., Luo W., Lin Q. (2016) A novel pathogenic bacteria (*Vibrio fortis*) causing enteritis in cultured seahorses, *Hippocampus erectus* Perry, 1810. *Journal of Fish Diseases* 39(6): 765–769.

Wilson M.J., Vincent A.C. (2000) Preliminary success in closing the life cycle of exploited seahorse species, *Hippocampus spp*., in captivity. *Aquarium Sciences and Conservation* 2(4): 179–196.