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THE EFFECT OF DISINFECTION ON SURVIVAL AND FEED QUALITY OF ROTIFERS (*BRACHIONUS PLICATILIS)* AND BRINE SHRIMP (*ARTEMIA SALINA*)

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ABSTRACT

Live feeds are used as an essential component during the larval stages of most marine finfish species in aquaculture. The microbial accumulation in batch culture of live feed has been identified as one of the major factors limiting the expansion of commercial farming of aquatic species in many countries. The main focus of the present study was to test the use of the antimicrobial agents bronopol (Pyceze) and Sanocare ACE for the disinfection of enriched rotifers and brine shrimp (*Artemia*) in a marine hatchery. The study also involved a feeding experiment with three different feeds in rotifer batch culture (Rocult, yeast and algae paste). The study has successfully tested disinfection protocols that were highly effective with regards to survival and feed quality of rotifers and brine shrimp. The results indicate that the use of 50 ppm bronopol for 2 hours is a very effective protocol for rotifer disinfection with negligible lethal effects on rotifers. A combination of 100 ppm bronopol and 600 ppm Sanocare ACE for 22 hours was a very effective disinfection protocol for brine shrimp with only moderate lethal effects on the brine shrimp. The commercial feed Rocult was the best feed in the feeding experiment but an inexpensive yeast diet proved almost as good.

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1 INTRODUCTION

Live feeds are used as an essential component during larval stages of most marine finfish species. The use of natural food such as marine rotifers *Brachionus* (figure 1) and brine shrimp *Artemia salina* (figure 2) was pioneered by Takashi Ito in Japan and since then live feed has been used as food for marine fish larvae (Korea-US-Aquaculture 2014). However, the production of suitable high quality live feed is still widely considered a serious bottleneck, limiting the expansion of commercial farming of aquatic species in many countries. Global statistics show that the mass-culture of many live feed species (such as plankton, brine shrimps, rotifers and marine worms) in hatcheries and grow-out operations is still one of the challenges faced in aquaculture (FAO 2012).

Figure 1: Female rotifer (*Brachionus plicatilis*) with one egg, from Korea-US-Aquaculture 2014.

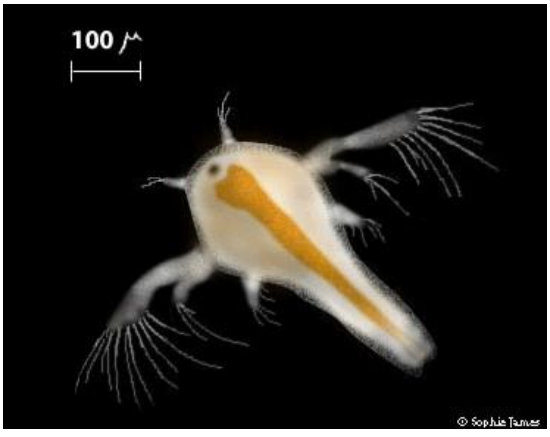

Figure *2:* Brine shrimp nauplius (*Artemia salina*), from Delbos and Schwarz 2009.

1.1 Background of marine larval culture in South Africa

South Africa is known to be one of the leading countries in abalone production. However, marine finfish production is still underdeveloped. A number of indigenous species, such as dusky kob (*Argyrosomus japonicus*), yellowtail (*Seriola lalandi*), white steenbras (*Lithognathus lithognathus*) and white stumpnose (*Rhabdosargus globiceps*) have been identified as suitable candidates for marine finfish aquaculture in South Africa (Hecht 2000). All these species and in fact most marine finfish depend on live feed during their larval stages.

The Department of Agriculture, Forestry and Fisheries: Directorate: Aquaculture Research and Development have an aquaculture research facility based at the Sea Point Aquarium in Cape Town, South Africa. One of the key objectives of the fish genetics and breeding subsection under the Directorate, is to supply the aquaculture industry with quality juveniles and first-class farming advice. The facility is presently conducting ongoing projects on dusky kob in order to produce quality eggs, larvae and juveniles to supply to local fish farms.

At present the facility uses females above 10 kg size with fecundity of about 1 million eggs per 10 kg fish or 100 000 eggs per kg female. Four females are induced per session and about 4-6 million eggs are spontaneously spawned between 32-72 hours after spawning induction using hormones. About 70 % of spawned eggs (~3.5 million) are on average fertilized, of which 90-95 % hatch. However, only about 15 % of post hatch larvae survive to the juvenile stage (complete metamorphosis and weaned = 28 mm). Hatching temperature is set at 22 °C and after hatching the temperature is increased to an optimum growth temperature of 25 °C (Collette *et al.* 2008). The initial hatching temperature is at the lower indicated setting to reduce larval deformities which are experienced when eggs are exposed to higher temperatures (>24 °C). The size of fertilized kob eggs is 0.9-1.0 mm in diameter. The larvae feed on live food at a length of 2.8 mm and approximately 3-4 days post hatch. In the hatchery the eggs are incubated at a density of 500 per litre. A newly hatched larva measures on average about 2.3 mm in length and yolk sac diameter is approximately 0.8 mm. Larvae are stocked at an initial density of 200 per litre. Weaned juveniles (28-31 mm) are reared at a density of 50 fish per litre and then grown to fingerlings (40 mm) at a density of 10 fish per litre. The nursery grows fingerlings to viable growers at 50 g body mass and at a production density of 20 grams per litre or 20 kg per cubic meter (Collette 2007).

Dusky kob is commercially produced by four companies in South Africa. The annual total productions of the four farms was 7,909 tons in 2011 (Department of Agriculture, Forestry and Fisheries 2012). This species is fed with microalgae (e.g. *Nannochloropsis oculata*, *Isochrysis* spp and *Pavlova lutheri*), rotifers (*Brachionus plicalitis*) and brine shrimp (*Artemia salina*) during the larval stage in South Africa. These live feed organisms can also be a primary source of pollutants entering the culture system (Davis 1990). It is believed that an excessive microbial load from live feed can cause massive mortalities of fish larvae since pathogens can be transferred from the live feed to the fish. Therefore, feeding of the fish larvae with live feeds represents a critical phase, especially considering the nutritional health of the fish.

1.2 Rotifer culture

1.2.1 The culture conditions for rotifers

The rotifer *Brachionus* spp. is the first live feed organism that has demonstrated acceptability for most marine species, and can typically be raised on a commercial scale (Delbos and Schwarz 2009). *Brachionus plicatilis* (L-strain) is a marine rotifer with a size range of 200-360 μm and is the most common live feed species in aquaculture (Delbos and Schwarz 2009). The culture of *Brachionus* spp. is influenced by various water quality parameters, namely temperature, dissolved oxygen (DO), pH and salinity. Rotifers of the genus *Brachionus* require oxygen concentrations above 1.0 mg/L for their growth, though some can tolerate anaerobic or near-anaerobic conditions for a short period of time. A salinity of 10-35 ppt and total ammonia nitrogen of ≤ 5 ppm are also recommended for the culture of this species (Korea-US Aquaculture 2014). They require a temperature range of 22 to 28 °C. The culture of this live feed is common due to some positive biological characteristics that include: small body size, round body shape, slow swimming speed, positive buoyancy, easy enrichment, high reproduction rate and high density cultures (Korea-US Aquaculture 2014).

1.2.2 Feeding and enrichment of rotifers

Rotifers are enriched with specially formulated feed products in order to boost their nutritional profile prior to feeding them to fish larvae (Delbos and Schwarz 2009). Activated baker's yeast has been used successfully as an inexpensive grow-out diet when fed at approximately 0.5 g/million rotifers/day (Delbos and Schwarz 2009). Baker's yeast has a small particle size (5-7 μm) and high protein content and is an acceptable diet for rotifers. When applied as a sole diet, it may support the mass production of rotifers in non-axenic (non-sterile) culture conditions where micro-organisms provide essential nutrients (Hirayama 1987). However, it is well known that yeast-fed rotifers lack the essential fatty acids required for the proper development and survival of several species of marine fish. Therefore, the yeast usually needs to be supplemented with essential fatty acids and vitamins to suit the larval requirements of the fish to be cultured (Hirayama and Satuito 1991).

Many commercial, artificial diets are available for feeding and enriching rotifers, such as DHA Protein Selco, S.parkle, S.presso, and S.tream. Rocult is another available off-the shelf dry rotifer diet belonging to the Larviva hatchery feed product line from Biomar in Denmark. Rocult is a formulation based on living yeast and algae that is high in protein and is nutritionally complete diet to grow rotifers in an optimal way. The feed is delivered in dried form which makes it easier to use and the daily production of rotifers is predictable and reliable (Larviva Biomar Hatchery 2014). Multigain also belongs to Biomar's Larviva product line and has been used as an enrichment feed for rotifers and brine shrimp. It has been designed to provide live feed with adequate nutritional composition. As a result, less sensitive and healthier larvae are produced with reduced deformity levels (Laviva Biomar Hatchery 2014). Concentrated algae paste is also widely used as a feed for growing rotifers. RotiGrow Plus is produced by Reed Mariculture and is a mixture of five different

microalgae, rich in highly unsaturated fatty acid (HUFA) and vitamins for rotifers culture. RotiGrow Plus is a clean, high yield rotifer feed that maximizes pre-enrichment levels of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA). It can also be used as a sole feed or in combination with other enrichments, depending on the nutritional requirements of the fish larvae (Reed Mariculture Inc. 2014).

1.2.3 Batch culture of rotifers

Over the years, several culture techniques have been used in the culture of rotifers. Among those, batch culture is the most commonly used method in rotifer production for marine fish hatcheries, probably because of its simplicity (Fukusho 1983, Nagata and Hirata 1986 and Snell 1991). This culture technique consists of either the maintenance of a constant culture volume with an increasing rotifer density or the maintenance of a constant rotifer density by increasing the culture volume (Fukusho 1983, Nagata and Hirata 1986 and Snell 1991). Although batch culture is simple, the most frequently reported problems associated with this system are: the unpredictability of rotifer mass production, the difficulty to manage and harvest large rotifer populations and the difficulty of producing clean rotifers that are free of floccules (suspended particles) and safe from a microbial point of view (Dhert *et al*. 2001). Jung (2012) has discovered that in the mass culture of rotifers, there are many factors affecting their population growth. Among them is the biological environment, such as contamination with bacteria and other organisms in the culture tanks. Copepods (*Tigriopus japonicus*, *Acartia*), ciliate protozoans (*Euplotes*, *Vorticella* and *Holosticha*) often co-exist in the marine *Brachionus* rotifer culture.

1.2.4 Rotifer culture contamination

The use of live feed is believed to be a major contributor to high mortalities of fish larvae as a result of contamination that may be traced to the live feed culture. Most bacteria are not pathogenic to rotifers, however, their proliferation must be avoided since their accumulation and transfer via the food chain can cause detrimental effects to fish larvae (Dhert 1996). Bacteria are always associated with mass production of rotifers and may cause an unexpected mortality or suppressed growth of rotifers. In some other cases, bacteria may cause no harm to the rotifers but infected rotifers may cause detrimental effects on fish larvae, resulting in poor survival and growth (Perez-Benavente *et al.* 1989).

1.3 Brine shrimp culture

1.3.1 The culture methods for brine shrimp

Among the live feeds used in the larval culture of fish and shellfish, nauplii and metanauplii of the brine shrimp (*Artemia*) constitute another widely used live prey item. Brine shrimp belong to the phylum Arthropoda, class Crustacea, like *Daphnia*, which is a copepod used as live food in the aquarium trade and for marine finfish and crustacean larval culture (Treece 2000). The cysts of brine shrimps are made available in storable cans and can be incubated for 24 hours in seawater. These cysts release

free-swimming nauplii that can directly be fed as a live food source to the larvae of a variety of marine as well as freshwater organisms, which makes them the most convenient, least labour-intensive live food available for aquaculture (Stappen 1996). Furthermore, the cultivation methods have gradually been optimized through the years and made more cost effective. Modern hatcheries now routinely disinfect the brine shrimp and use decapsulated cysts to reduce labor and microbial loads. Various brine shrimp biomass preparates are also being applied in hatcheries, nurseries and broodstock rearing (Stappen 1996). Decapsulation is an easy method that is used to remove the outer layer of the shell prior to incubation of the cysts (Gilbert and Watson 2006). Aside from eliminating the shell, decapsulation sterilizes the cysts and can increase hatch rates if done properly (Gilbert and Watson 2006). Sep-Art is a new technology that provides a magnetic coating on brine shrimp cysts. According to the producer, this new technology is highly efficient and allows a fast and complete separation of nauplii and unhatched cysts (Ocean Nutrition 2014).

1.3.2 The culture conditions for brine shrimp

Both temperature and salinity significantly affect the survival and growth of brine shrimp, with the effect of temperature being more pronounced. A broad range of temperatures and salinities meet the requirements for >90 % survival. Strains from thalassohaline (hypersaline) environments have an optimal temperature range of 20-25 °C where mortalities are <10 %. Interaction between temperature and salinity is limited; substantial differences in tolerance have been recorded at low salinities (around 5 g l^{-1}) and high temperatures (30-34 °C). At elevated temperatures the survival of the Great Salt Lake (GSL) strain is significantly higher than for other strains (Stappen 1996).

1.3.3 Feeding or enrichment of brine shrimp

The nutritional value of brine shrimp can be improved to suit the predator's requirements by feeding specific amounts of particulate or emulsified products that are rich in highly unsaturated fatty acids (HUFA) to the nauplii. Application of this method of bio-encapsulation, is also called enrichment or boosting. This method of enriching brine shrimp has had a major impact on improved hatchery outputs, not only in terms of survival and growth of many species of fish and crustaceans, but also with regard to their quality that includes reduced incidence of malformations, improved pigmentation and stress resistance (Stappen 1996). Like rotifers, brine shrimp act as ideal carriers that carry nutrients to fish larvae. They do, however, need to be enriched with nutrional diets that are essential for the growth and survival of fish larvae. SELCO (Self-Emulsifying Lipid Concentrate) is an enrichment product that was developed by "Artemia Systems NV". This product has been the benchmark *Artemia* enrichment formula in the aquaculture industry. SELCO is rich in Highly Unsaturated Fatty Acids (HUFA's) and contains particularly high levels of the essential Omega-3 fatty acids, EPA and DHA. This diet has been shown to increase both the survival and the growth of marine fish larvae according to Brine Shrimp Direct (2014).

1.3.4 Brine shrimp culture contaminations

It has been demonstrated that bacteria can have a beneficial effect during brine shrimp culture (Verschuere *et al*. 1999). Studies also show that some bacterial strains may improve the survival and growth rates of brine shrimp. Similarly, the culture of brine shrimp under non-sterile conditions usually results in a higher biomass production than that under sterile conditions (Verschuere *et al*. 1999). Diseases can be problematic in batch culture systems of brine shrimp. For example, some species of *Vibrios* have been identified as harmful organisms that may dominate the flora of brine shrimps and rotifers (Munro *et al*. 1999). Reduction of this harmful bacterial load on live food organisms is important for achieving good survival and growth of fish larvae.

1.4 Use of antimicrobial agents in live feed culture

The microbial accumulation in batch culture of live feed has been identified as one of the major biochemical factors affecting the culture of marine finfish and molluscs. A number of methods have been developed for reducing the bacterial load of live feed, e.g. UV irradiation or Pyceze treatment (Novartis 2014). Antibacterial substances such as Pyceze (50% bronopol) have been reported to be effective against bacterial proliferation over a wide pH range (Bryce *et al*. 1978). Sanocare ACE (*Artemia* Condition Enhancer, INVE Aquaculture) has also been used as an antibacterial agent and a water conditioner for improved brine shrimp quality. Sanocare ACE increases the quality and vitality of hatched, concentrated and stored *Artemia* nauplii. It improves water quality conditions during enrichment, stabilizes pH levels and avoids foaming during hatching, enrichment and storage (INVE Aquaculture 2014). Sanocare ACE was the first product in the aquaculture industry able to reduce the development and transfer of putative pathogens associated with live food cultures. Sanocare ACE is a self-emulsifying product that keeps bacterial levels in brine shrimp hatching water at a very low level. The use of it has been found to reduce the *Vibrio* load up to 10,000-100,000 fold (INVE Aquaculture 2014).

1.5 The objectives of the study

The main aim of this study was to determine suitable disinfection techniques for the production of rotifers and brine shrimp for fish hatcheries. A vital aspect of the study was to analyse the effect of disinfection on the viability of the prey themselves. A secondary aim was to compare the suitability of different foods for rotifer batch culture. Specific objectives of the experiment include:

A) To compare the use of different feeds (Rocult, yeast and algae paste) for rotifers to respectively monitor their fertility and population growth.

B) To analyse the effect of Pyceze disinfection on rotifer viability and microbial load.

C) To analyse the effect of Pyceze and/or Sanocare ACE disinfection on brine shrimp viability and microbial load.

2 MATERIALS AND METHODS

2.1 Location and dates of the experiments

All experiments were performed at the Marine Research Institute (MRI) aquaculture facility in Staður, in Grindavík Iceland from the 12th to the 22nd January 2014. Rotifers, brine shrimps, feeds, equipment, chemicals, transport and assistance were provided by the facility.

2.2 Density determination

The density of live feed was counted by obtaining samples (0.25 ml for rotifers and 0.5 ml for brine shrimp) from each experimental container. The sample was put in a test tube and 1 drop of Lugol's solution (5 % solution) added to kill and stain the rotifers (Delbos and Schwarz 2008). The samples were then diluted with tap water and placed on an s-shaped tray used for live feed counting (figure 13 in the appendix). The tray was placed under a light microscope for counting of individual animals at 40 x magnification. The total density of prey animals was determined from the counting as well as the percentage of egg-bearing rotifers. The counting was done by sampling each individual tank and calculating the mean for each duplicate set.

2.3 Rotifer trials

2.3.1 Feeding trial

A 3 x 3 setup of 25 L black, conical tanks (silos) was prepared (figure 3) and the silos inoculated with rotifers for the experiment. The silos were aerated with pure oxygen to ensure a continuous supply of dissolved oxygen. The water temperature was kept between 25-27 ˚C and the salinity was stable at 32 ppt. All the tanks were exposed to light throughout the experiment. Sponge filters were submerged in the tanks to trap both large pieces of suspended matter and protozoans. The sponges were washed with high pressure water on a daily basis.

The trial was designed to compare the following three feed products:
A = Rocult, Producer: Biomar, DENMARK.
B = Semi dry yeast, Producer: Angel Yeast Co. Ltd, CHINA.
C = Algae paste (Rotigrow Plus), Producer: Reed Mariculture Inc, USA.
The initial stocking density was ≈1000 rotifers/ml in each silo. The three sets of rotifer tanks were fed with different diets (A, B and C, respectively) for a period of four days. Feeds A and B were supplied at 0.45-0.7 g/million rotifers/day, feed C was supplied at 1.5 ml/million rotifers/day. The feeds were supplied in 8 equal rations per day by automatic pumps at rate of 125 ml per 3 hours. The population density and egg percentage were measured daily after feeding. About 4 litres of water were flushed from the silos each morning to harvest rotifers and create space for the new feed.

Figure 3: Rotifer silos used for the feeding trial experimental set up, with triplicate silos for each type of feed diet. The picture was taken by author at Staður in January 2014.

The feeds were used in different quantities as shown in table 1 below.

Table 1: The table below shows the different feeds, quantities and the ClorAm-X used to mix with individual feed during the rotifer feeding trial.

Days	Feed type	Feed Quantity (g)	ClorAm-X (g)
	Rocult	39	35,0
	Yeast	39	35,0
1	Algae paste	87 ml	34,8
	Rocult	48	19,2
	Yeast	42	16,8
2	Algae paste	96 ml	38,4
	Rocult	42	16,8
	Yeast	42	16,8
3	Algae paste	87 ml	34,8
	Rocult	60	24,0
	Yeast	60	24,0
4	Algae paste	120 ml	48,0

The density of rotifers was measured daily after feeding different diets for four days. The specific growth rate was calculated using the equation described by Rombaut *et al*. (1999):

$\mu = (\ln N_t - \ln N_0)/t$,

Where: μ=specific growth rate, N_t =rotifer density after culture period t (individual's ml^{-1}), N_0=initial rotifer density (individuals ml^{-1}), t=culture period (day).

Feeds A and B were prepared by mixing the total daily ration of feed mixed with 40 % ClorAm-X (90 % during first day) in about 300 ml warm water in a blender for 2 minutes. Feed C was mixed gently with ClorAm-X in 300 ml of seawater. ClorAm-X

is a dry powder substance used as water conditioner for detoxification and removal of ammonia, chlorine and chloramines in both fresh and salt water. All feed for each triplicate set of silos was stocked in 3 L cylinders under constant aeration and connected to the automatic pumps. Feeding was adjusted daily depending on the population density counted.

2.3.2 Rotifer disinfection trial

The disinfection trial was performed in 6L transparent plastic cylinders. Six duplicate sets (figure 4) of cylinders (6 x 2) were inoculated with rotifers obtained from the master cultures at a stocking density of ≈ 2000 rotifers/ml in a total volume of 6L. The cultures were all aerated with pure oxygen and enriched with Multigain (Biomar, Denmark) at 0.2 g/million rotifers. Temperature and oxygen were measured in all cylinders. Pyceze (50 % bronopol) was added at two different dosages for two different time intervals as shown below (table 2). One set of cylinders served as a control group and did not receive any Pyceze treatment. Pyceze is a clear fluid bath additive that contains 50 % bronopol. Bronopol (2-bromo-2-nitropropane-1,3-diol) is a common name for the active ingredient in a series of broad spectrum antimicrobial agents and it is marketed by The Dow Chemical Company and its global affiliates under several trade names. Pyceze is one of the products containing bronopol and it is available as a white to off white crystalline solid powder. Pyceze is produced by Novartis Animal Health UK Ltd as a bactericide and for treatment and control of fungal infections (Novartis Animal Health 2014).
To prepare a 50 ppm bronopol concentration, 600 ml water were mixed with 0.6 ml Pyceze (100 mg/L) and added to the 6 L cylinder containing about 5.4 L culture water. The dosage of the chemical was halved (i.e. 0.3 ml Pyceze) to prepare the 25 ppm solution. The density was counted after 2 hours of sterilization and again after 6 hours. These time intervals were selected due to the recommended enrichment period for rotifers by the suppliers. Rotifers are normally enriched for 2 - 6 hours with Multigain (Larviva Biomar Hatchery 2014). To facilitate a direct comparison of treatment effect the initial density was normalised to 2000/ml and subsequent densities were adjusted accordingly. The normalisation factor (NF) was calculated from actual density (AD) according to: NF = 2000/AD. Normalised density (ND) was calculated from actual density (AD) according to: ND = AD x NF.

Samples were collected at the end of each time interval for microbial analysis. Collection was done by filtering about 3 L of culture medium on a fine filter (64 micron mesh) and collecting the sludge in 10 ml glass bottles. The equipment was sterilized before sampling from each cylinder. It was first washed in a Virkon-S solution, a disinfectant chemical that kills about 99.99 % of numerous pathogens including 31 bacterial strains, 58 viruses and six fungi with no evidence of resistance. The equipment was then rinsed with seawater and finally washed in water containing vinegar for the removal of excess chlorine. Seawater was used to wash the rotifers collected on the mesh sieve to remove suspended solids on the cultures. The sludge was frozen immediately (-20 °C) and taken for microbial analysis at Matís some days later. Matís Ltd. Icelandic food and biotech R&D company is a government owned, independent research company that pursues research and development aligned to the

food and biotechnology industries as well as providing analytical testing service for public and private authorities.

Figure 4: The rotifer cylinders showing a 6 x 2 setup for the rotifer disinfection trial, picture taken by author in January 2014.

Pyceze (50 % bronopol) was used to disinfect rotifers at different concentrations and durations as described below. Each group included a duplicate set of tanks.

Table 2: The quantities of disinfectant used for the rotifer disinfection trial.

Tank No	Treatment	Dosage applied	Duration
1A	Control	0 mg/L	2 hours
1B	Control	0 mg/L	2 hours
1A	Control	0 mg/L	6 hours
1B	Control	0 mg/L	6 hours
2A	Pyceze (50% bronopol)	50 mg/L (25 ppm)	2 hours
2B	Pyceze (50% bronopol)	50 mg/L (25 ppm)	2 hours
2A	Pyceze (50% bronopol)	50 mg/L (25 ppm)	6 hours
2B	Pyceze (50% bronopol)	50 mg/L (25 ppm)	6 hours
3A	Pyceze (50% bronopol)	100 mg/L (50 ppm)	2 hours
3B	Pyceze (50% bronopol)	100 mg/L (50 ppm)	2 hours
3A	Pyceze (50% bronopol)	100 mg/L (50 ppm)	6 hours
3B	Pyceze (50% bronopol)	100 mg/L (50 ppm)	6 hours

2.4 Brine shrimp trials

2.4.1 Brine shrimp disinfection trial

The *Artemia* cysts used in the study originated from the Great Salt Lake (GSL) in Utah (INVE Aquaculture 2014). Non-decapsulated cysts were inoculated at 500 cysts/ml as a part of the hatcheries routine production. Sanocare ACE (0.5 g/L) and bronopol (0.1 g/L) were added to the inoculation to enhance water quality and reduce microbial levels. After hatching, the nauplii were separated from the cysts and washed

with clean seawater before being stocked into the experimental tanks. A set of six duplicate (6 x 2) 6 L tanks (transparent, plastic cylinders) were inoculated with *Artemia* nauplii from the hatched cysts at a stocking density of ≈ 300 nauplii/ml. All groups were immediately enriched with A1 DHA Selco (INVE Aquaculture 2014) in one 200 ppm ration. The anti-microbial agents were added immediately to all except one duplicate set of tanks at different concentrations and different time intervals as shown below (table 3). Samples were collected after 10 and 22 hours and stored in a freezer (-20 °C). The time intervals for sampling were selected based on the enrichment period of brine shrimp, which has been recommended to be 12–24 hours (INVE Aquaculture 2014). Samples collection was done following the same method as rotifers which is described under section 2.3.2.

Table 3: The quantities of disinfectants used for brine shrimp disinfection trial. Pyceze and/or Sanocare ACE were used at different concentrations to disinfect brine shrimps at different time periods.

Tank No	Treatment	Dosage applied	Duration
1A	Control	0 g/L	10 and 22 hours
1B	Control	0 g/L	10 and 22 hours
2A	Sanocare	0.6 g/L	10 and 22 hours
2B	Sanocare	0.6 g/L	10 and 22 hours
3A	Sanocare + Pyceze	0.6 g/L + 0.2 g/L	10 and 22 hours
3B	Sanocare + Pyceze	0.6 g/L + 0.2 g/L	10 and 22 hours
4A	Sanocare + Pyceze	0.6 g/L + 0.4 g/L	10 and 22 hours
4B	Sanocare + Pyceze	0.6 g/L + 0.4 g/L	10 and 22 hours
5A	Pyceze	0.2 g/L	10 and 22 hours
5B	Pyceze	0.2 g/L	10 and 22 hours
6A	Pyceze	0.4 g/L	10 and 22 hours
6B	Pyceze	0.4 g/L	10 and 22 hours

2.5 Microbial analysis

The assessments were done by pipetting 1 ml of sample to carry out three dilutions (10^1, 10^2,10^3) to be inoculated on plates. The dilutions were transferred to a petri dish containing a Plate Count Agar (PCA, Biokar Diagnostic cod. BK 144HA, Beauvais, France) with 2 % NaCl. This growth medium, or culture medium, is a liquid or gel that is designed to support the growth of microorganisms or cells under specific incubation environment. Another 1 ml sample was also inoculated in a plate media (10^0) and all the plates were incubated for 72 hours at 30°C. After three days the plates were counted for total colony forming units under a microscope (De Donno *et al*. 2010).

The microbial counts were done by counting total number of colonies in each plate. Only the best plates (with countable colonies) were selected for the microbial counts. The 10^3 dilution plate was selected for this study since the other plates contained more growth which was difficult to count. The total colonies counted on each plate were multiplied by 1000 to account for the 10^3 dilution.

2.6 Water quality parameters

Water temperature and dissolved oxygen were measured on a daily basis using an electronic multimeter, while the salinity was measured using a digital handheld refractometer on a daily basis.

2.7 Statistical analyses of data

Data sets are presented as mean ± standard error. The data was subjected to one-way and two way analysis of variance (ANOVA) using Excel software in order to determine the significant differences among treatment means at $p < 0.05$.

3 RESULTS

3.1 Feeding of rotifers

3.1.1 Effect of different feeds on population density of rotifers

The initial density of rotifers in all the silos was ≈1000 rotifers per ml. Yeast produced the highest population density on the second day but Rocult produced the highest density from the third to the last day (figure 5). A significant difference was found between the feeds ($p = 0.002$), 1-way ANOVA. No difference was found between Rocult and yeast ($p = 0.745$) but algae paste was different from both Rocult ($p = 0.003$) and yeast ($p = 0.014$), according to Tukey Test.

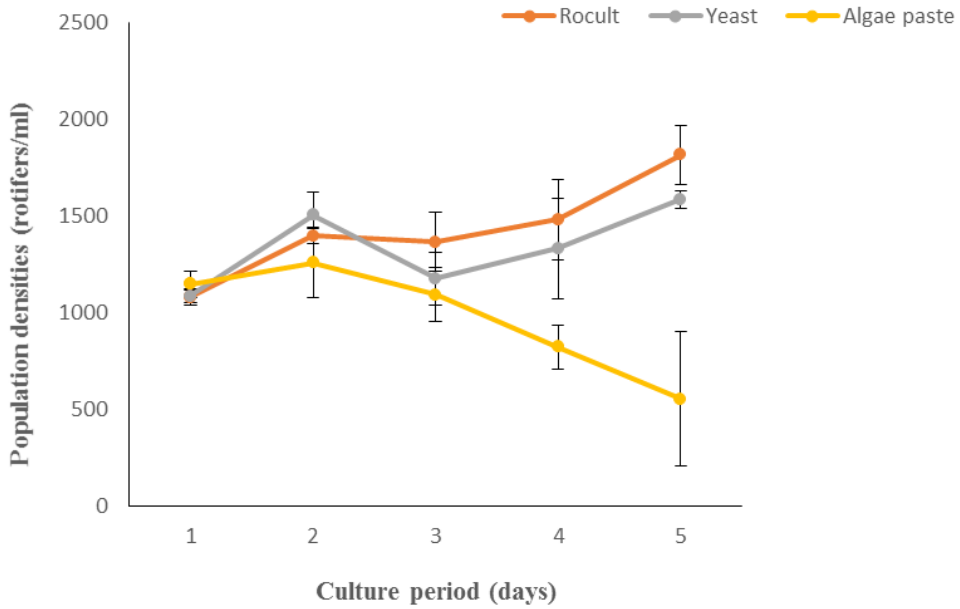

Figure 5: Rotifer population densities counted on a daily basis based on different feeds. Each data point represents the average population density of rotifers on each day and the error bars represent the standard error of the mean.

3.1.2 Rotifer production and harvesting

The production from the rotifer batch system is ultimately contributed from the combined population increase and the daily harvest of rotifers. During the feeding trial an average total of 24.3 million rotifers was harvested from each Rocult tank, which resulted in the average total production of 42.6 million rotifers/tank or 0.43 million rotifers/L/day. An average total of 22.4 million rotifers was harvested from each yeast tank, resulting in an average total production of 0.35 million rotifers/L/day. An average total of 12.7 million rotifers was harvested from each algae tank, resulting in an average total production of 0.17 million rotifers/L/day. The daily production of rotifers was thus highest on the Rocult diet but lowest on the algae paste diet (figure 6). There was a significant difference found between Rocult and algae paste ($p < 0.05$) but no differences were observed between Rocult and yeast, or yeast and algae paste ($p > 0.05$), Fisher LSD.

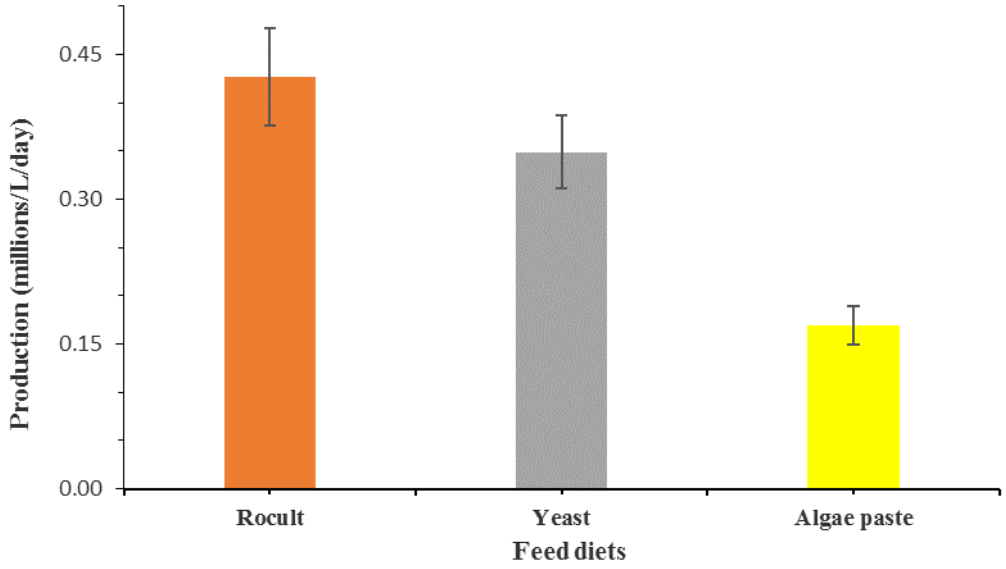

Figure 6: The average daily rotifer production from the three feeds in the rotifer feeding trial. Each column represents the average daily rotifer production from each feed diet. The error bars indicate the standard error of the mean.

3.1.3 Egg percentage

Figure 7 shows the development of the rotifer egg percentage during the feeding trial.

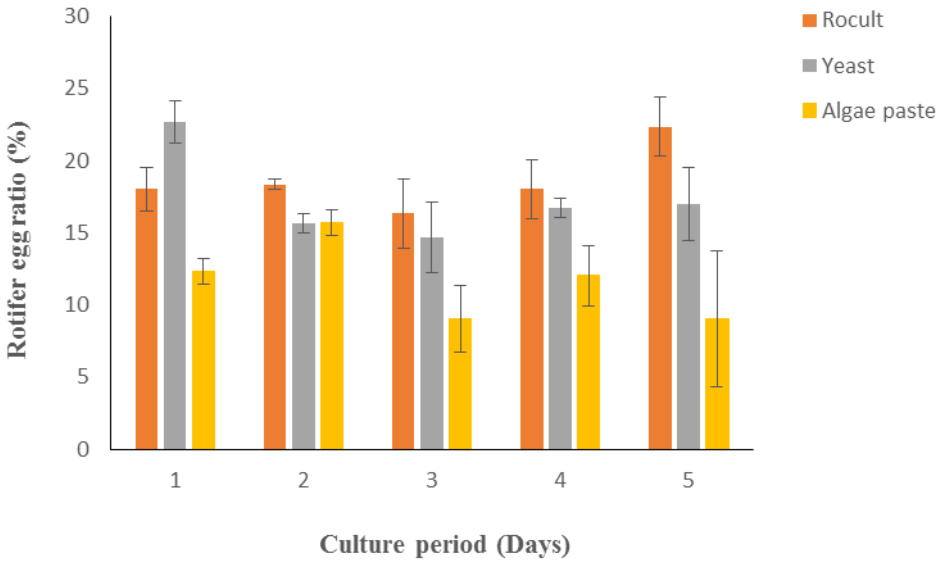

Figure 7: The percentage of pregnant rotifers (egg carriers) during the feeding trial. Each column represents the daily mean egg percentage from each feed diet each day. The error bars indicate the standard error of the mean.

The percentage of egg carrying rotifers fed with Rocult was relatively stable throughout the experiment but increased by 22 % during the last day of the experiment. The percentage of egg carrying rotifers fed with yeast was high initially but decreased during the second day and remained relatively low and stable. The egg percentage of algae paste fed rotifers was low and variable throughout the experiment

(figure 7). The percentage of egg carrying rotifers was less than 25 % in all groups throughout the experiments, while a 30 % percentage is considered desirable during the culture of rotifers (Delbos *et al.* 2001). A significant difference was found between feeds ($p = 0.0001$), 1-way ANOVA. No difference was found between Rocult and yeast fed rotifers ($p = 0.663$) but algae paste was different to both Rocult ($p = 0.000$) and yeast ($p = 0.001$), Tukey Test.

3.1.4 Specific population growth rates

The specific population growth rates (SPGR) during the feeding trial were calculated on a daily basis and proved highly variable between days (figure 8). The SPGR of the Rocult group varied between 14 % and 39% per day and averaged 25.9 % during the feeding trial. The SPGR of the yeast group was only slightly inferior and averaged 22.6 % during the feeding trial. The algae paste group had negative growth rates on the last two days, consistent with a culture crash and thus the last day was excluded from calculations of SPGR. Still the average SPGR of this feed group was 0 % during the four day period, so clearly this feed was not suitable as used in this trial. The results indicate that Rocult was the best feed in the trial, with yeast as a close second.

The daily specific growth rates during the trial are shown in figure 8 and the average SPGR's during the entire trial are shown in figure 9. The growth rates were based on the daily production levels (production = harvest + population increase) and calculated using the formula described by Rombaut *et al*. (1999). There was a significant difference between rotifer specific population growth rates with respect to the type of feed diet ($p = 0.04$). The Tukey Test showed no significant differences between Rocult and yeast ($p = 0.985$); Rocult and algae paste ($p = 0.066$) or rotifers and algae paste ($p = 0.075$). However, the Fisher's Least Significant Difference test (LSD) showed a significant difference between Rocult and algae paste ($p = 0.027$); yeast and algae paste ($p = 0.030$) but no significant difference between Rocult and yeast ($p = 0.871$).

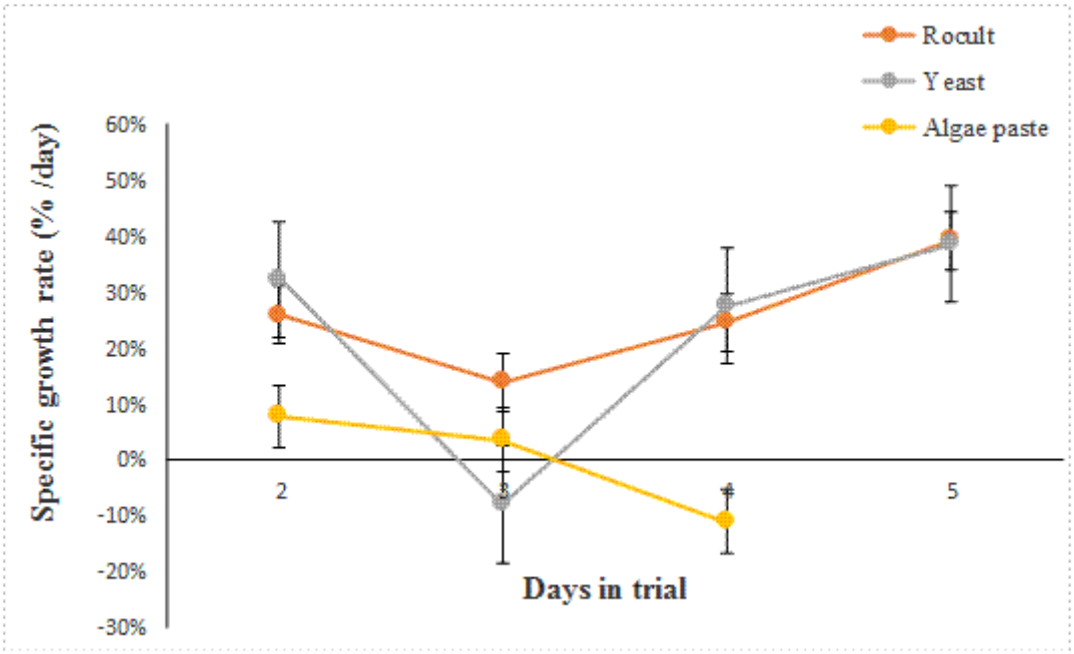

Figure 8: Specific population growth rates of rotifers observed on a daily basis during the feeding trial. The data points represent the daily average growth rates of rotifers. The last day with algae paste was excluded from the graph due to a culture crash. The error bars indicate the standard error of the mean.

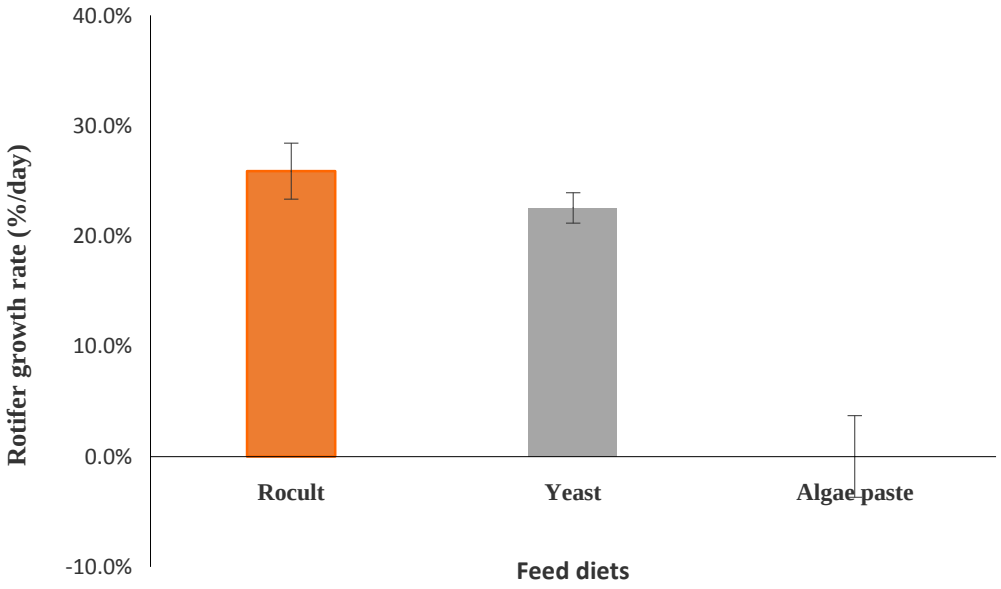

Figure 9: Average daily growth rates of rotifers during the entire feeding trial. The last day with algae paste was excluded from the calculation due to a culture crash. The error bars indicate the standard error of the mean.

A significant difference was found between the three feed types ($p < 0.05$), one-way ANOVA. The Tukey test further showed a significant difference between Rocult and

algae paste ($p < 0.05$) and between yeast and algae paste ($p < 0.05$). However, there was no difference observed between Rocult and yeast ($p > 0.05$).

3.1.5 Effect of water quality parameters

Oxygen levels were found to be the most important contributing environmental factor affecting the population density of rotifers during the culture period. A decline in oxygen levels apparently caused a massive collapse (42-92 % mortality) in culture tanks fed with algae paste on the fifth day as shown in table 4. Salinity is also suspected to be another factor affecting the population growth of rotifers. It is possible, although unlikely, that the gradual reduction in salinity levels may have affected the population growth in tanks since all the feeds were mixed with fresh water. An accidental overdosing of ClorAm-X into the algae paste tanks might also have contributed to inferior water quality in those culture tanks.

3.2 Disinfection of rotifers

3.2.1 Effects of disinfection on rotifer densities

The population densities in all the experimental groups were not affected by a 2 hour disinfection with Pyceze (bronopol), while a 6 hours treatment resulted in reduced densities (figure 10). The initial density of the control groups was only slightly reduced after 6 hours, but the density of the treatment groups showed a huge negative effect between 2 and 6 hours of treatment. There was no significant difference observed after 2 hours on neither the concentrations ($p > 0.05$). However, there was a slight difference between the control and 25 ppm ($p = 0.05$), also between the control and 50 ppm ($p = 0.05$) at 6 hours according to Fisher (LSD). Treatment duration had a stronger effect on the densities as shown after 6 hour of treatment. Even the lower concentration (25 ppm) is too high for a 6 hour duration as 50 % losses are unacceptable.

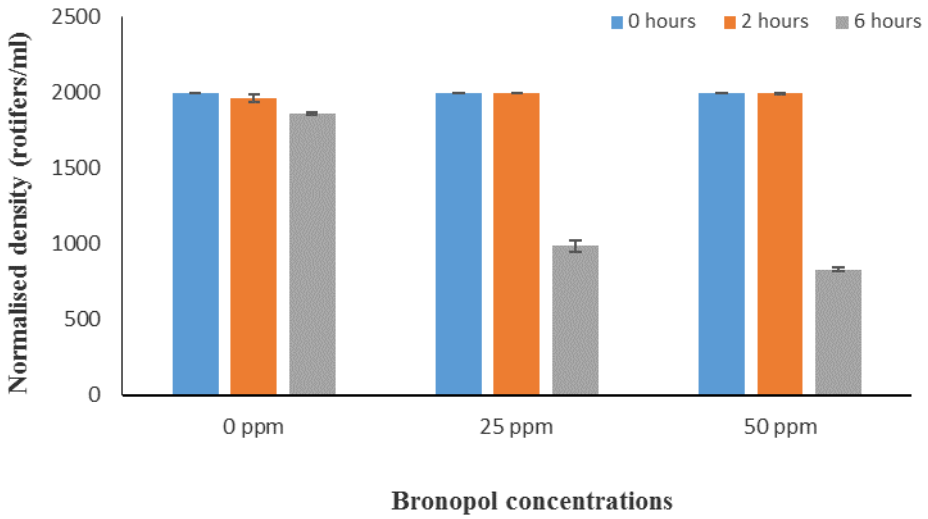

Figure 10: Rotifer population densities before and after treatment with Pyceze (bronopol). The columns represents the normalized density of rotifers. The error bars represent the standard error of the mean.

Table 4 below shows that the control tanks had a density effect of only -2 % after 2 hours and -7 % after 6 hours of treatment. The densities in both the treatment groups were almost unaffected after 2 hours of treatment but a massive decline of -51 and -58 % was observed after 6 hours of treatment in both groups.

Table 4: Population density changes (in %) during the rotifer disinfection trial.

Treatment	2 hours	6 hours
Control (no treatment)	-2 %	-7 %
25 ppm bronopol	0 %	-51 %
50 ppm bronopol	0 %	-58 %

3.2.2 Effect of disinfection on total microbial counts from rotifer cultures

The microbial load on individual rotifers was calculated based on the assumption of 300,000 rotifers/ml sample or 300 rotifers/µl sample (1000-fold dilution). The microbial loads in CFU per rotifer are presented on a linear scale to quantify the relative treatment effects. Figure 11 shows that the effectiveness of the disinfection treatments was both duration- and concentration dependent and the total microbial counts showed a decreasing mean trend with both concentration and duration in all the groups. The microbial counts were found to be 1300 CFU/rotifer after the 2 hours and 1000 CFU/rotifer after 6 hours for the controls. The 25 ppm treatment showed about 890 CFU after 2 hours of treatment and about 660 CFU after 6 hours of treatment. The 50 ppm concentration showed microbial counts of about 710 CFU after 2 hours of treatment and about 520 CFU after 6 hours of treatment (table 11).
The microbial counts were generally about 25 % lower after the longer treatment and also about 25 % lower from the higher concentration. This is not a large difference in effectiveness and in view of the lethal effects of the 6 hour treatment, it can be

concluded that the 2 hour at 50 ppm bronopol treatment was the best protocol tested. This protocol reduced the microbial load per rotifer by 30-40 % as compared to controls, without any negative effects on rotifer viability.

A one-way-ANOVA showed a significant effect of treatment duration and treatment concentration ($p = 0.016$ and 0.003, respectively,). There was a difference observed when comparing both 25 ppm and 50 ppm with controls ($p = 0.039$ and $p = 0.030$, Tukey's Test). However, there was no statistical difference observed between the 25 ppm and 50 ppm concentrations ($p = 0.079$). There was no significant effect of treatment concentrations ($p = 0.355$) after 6 hours of treatments.

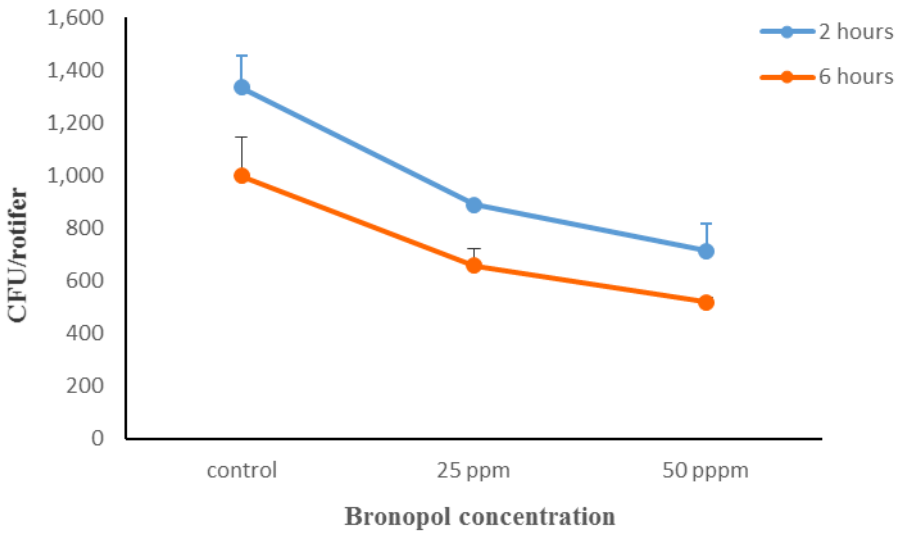

Figure 11*:* Total microbial counts (CFU per rotifer) after 2 and 6 hours of treatment in the rotifer disinfection trial. Each data point indicates the number of colonies counted per individual rotifer. The error bars represent the standard error of the mean.

3.3 Culture of brine shrimp

3.3.1 Effect of disinfection on brine shrimp densities

Figure 12 shows the population densities of brine shrimp before and after 10 and 22 hours of treatment with different combinations of Sanocare ACE (S) and Pyceze (P). After 10 hours of treatment with S and/or P, the densities in all treatment groups were significantly reduced by about 20 % ($p < 0.05$). The final density in group S was, however, restored to the initial level after 22 hours, suggesting that the counting after 10 hours was inaccurate. The densities in control and S group were not significantly affected after 22 hours ($p > 0.05$), however, the controls were significantly different from P200 and P400 after 22 hours ($p < 0.05$). S+P200 and S+P400 were also significantly different from P200 and P400 after 22 hours ($p < 0.05$). The group notations are explained in the caption of figure 12. It is important to keep in mind that Pyceze (P) includes 50% active bronopol.

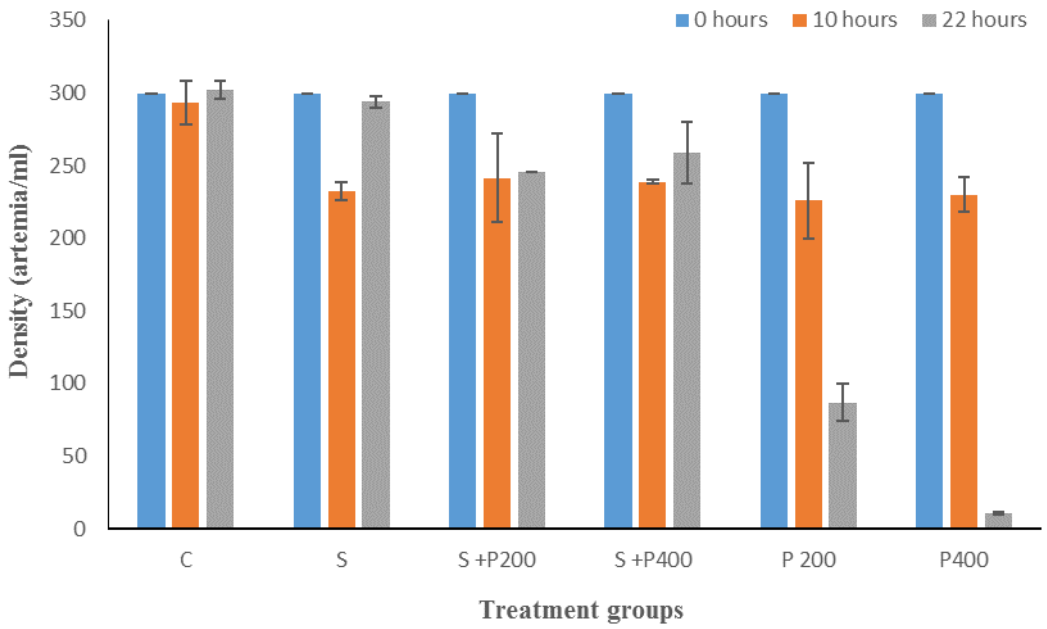

Figure 12: Population densities of brine shrimp disinfected with Sanocare ACE (S) and Pyceze (P). P200 and P400 mean 200 ppm and 400 ppm Pyceze, respectively (100 ppm and 200 ppm active bronopol, respectively). The error bars represent the standard error of the mean.

After 22 hours of treatment the densities of groups S+P200 and S+P400 were still reduced by about 15-20 % from the initial level but the densities of both groups P200 and P400 had shown a massive decline. The density in group P200 had been reduced by -71 %, while group P400 had crashed almost entirely (-96 %). This shows that the combination of Pyceze and Sanocare ACE has a much milder effect on population densities as compared to Pyceze only, which proved lethal after 22 hours of treatment. A one-way ANOVA revealed a significant duration-dependent effect on population densities before and after treatment with Pyceze and Sanocare ACE ($p < 0.05$). Table 5 shows a review of the relative density effects from the different treatments.

Table 5*:* Population density changes in brine shrimp disinfected with Sanocare ACE (S) and Pyceze (P). P200 and P400 include 100 ppm and 200 ppm active bronopol, respectively.

Treatment	10 hours	22 hours
Control (no treatment)	-2 %	1 %
S 600 mg/L	-23 %	-2 %
S 600 mg/L + P 200 mg/L	-20 %	-18 %
S 600 mg/L + P 400 mg/L	-20 %	-14 %
P 200 mg/L	-25 %	-71 %
P 400 mg/L	-23 %	-96 %

The survival rates are also shown in table 10 in the Appendix. Population density changes over time are illustrated in Table 5.

3.3.2 Effect of disinfection on total microbial counts from brine shrimp cultures

The microbial load of individual brine shrimp was calculated based on the assumption of 30,000 brine shrimp/ml sample or 30 brine shrimp/µl sample (1000-fold dilution). The microbial loads in CFU/brine shrimp were presented on a linear scale to quantify the relative treatment effects (figure 13).

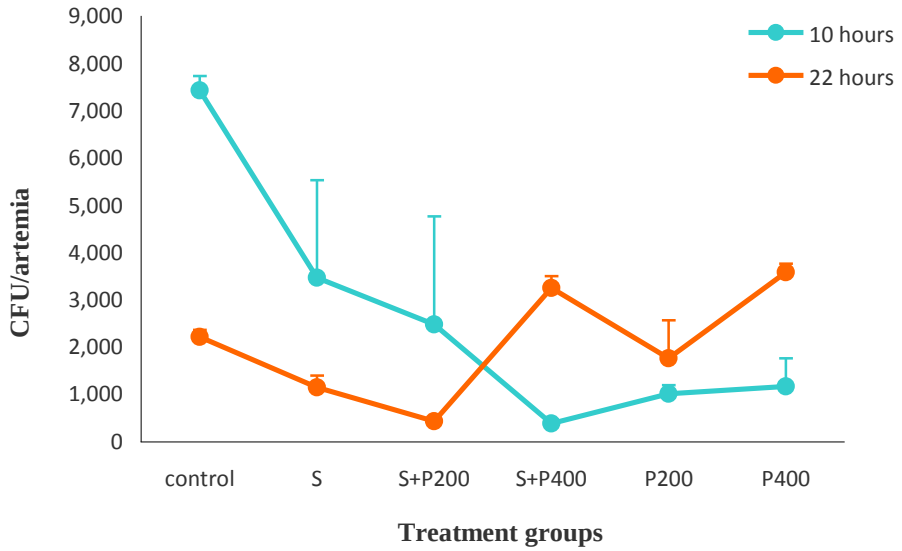

Figure 13: Total microbial counts (CFU per brine shrimp) in the brine shrimp disinfected with Sanocare ACE (S) and Pyceze (P). P200 and P400 mean 200 ppm and 400 ppm Pyceze, respectively (100 ppm and 200 ppm active bronopol, respectively). The error bars represent the standard error of the mean.

Figure 13 shows that both the antimicrobial agents tested were highly effective and also positively synergistic in their effect. The effectiveness was also quite duration dependent and the 10 and 22 hour treatments produced quite different results. After 10 hours all treatments were highly effective but the S+P400 combination was extremely effective with only 383 CFU/ brine shrimp (-95 % effect). The P200 and P400 treatments were also very effective with CFU's of 1000-1200/ brine shrimp.
After the 22 hour treatment, the situation was, however, quite different. The CFU's were now higher for the previously lowest treatments (S+P400, P200, P400), most likely due to induced mortality from the treatments. The S treatment was now very effective (1150 CFU/ brine shrimp) but the S+P200 treatment was extremely effective with only 433 CFU/ brine shrimp. There was therefore a clear synergistic effect of both the chemicals used and it can be concluded that the S+P200 for 22 hour treatment was the best protocol tested. This protocol reduced the microbial load on the brine shrimp by 95 % as compared to initial controls, without only moderate effects on brine shrimp viability.

No significant differences were observed after 10 hours of treatment ($p > 0.05$, one-way-ANOVA). A one-way-ANOVA did not show significant differences between treatments after 22 hours ($p = 0.138$). However, Fisher LSD showed significant differences between S+P200 and P400 ($p < 0.05$) and also between S+P200 and S+P400 ($p < 0.05$).

4 DISCUSSION

Despite the improved technology in the batch culture of rotifers (Dhert *et al.* 2001 and Yoshimura *et al*. 2003), poor performance is still a common problem in aquaculture hatcheries. Many factors have been linked to rotifer growth performance, such as nutrition and different microorganisms. The bacterial communities associated with rotifers might be one of the most important factors affecting the culture of rotifers.

In the feeding trial, rotifers fed with Rocult showed the best daily production, best fertility and best growth rates as compared to rotifers fed with yeast and algae paste. Therefore Rocult was considered to be the best feed diet for rotifers. The Rocult diet may have performed best because it is made up of live yeast that is rich in proteins and green algae that provide necessary vitamins to rotifers. Yeast, however, performed almost as well as Rocult and much better than the algae paste. The density decline on the second day with yeast may have been caused by overfeeding. The poor performance of rotifers in tanks fed with algae paste may also be the result of the amount of feed provided. Higher food quantities may result in higher mortalities of rotifers while insufficient food may result in poor growth rates as a result of competition between live feed and protozoans. The population densities of rotifers are known to be affected by the amount of food given to the animals on each day (Theilacker and McMaster 1971). It is also possible that the poor performance from the algae paste fed rotifers may have been caused by an overdosing of ClorAm-X added while mixing the feeds. There was also a massive decline in oxygen levels on the last day, which was found to be the most contributing factor to high mortalities in the algae paste tanks.

Rotifers are mostly cultured in batch culture systems that generate highly variable conditions, which can influence growth performance and the composition of microbial communities (Rombaut *et al*. 2001). High rotifer densities demand high feeding levels and produce high concentrations of waste products and thus creating high loads of organic material that is utilized as a feed source by bacteria. These intensive rearing conditions allow heterotrophic bacteria to grow fast in culture tanks (Skjermo *et al*. 1997, Verschuere *et al*. 1997, Skjermo and Vadstein 1999). Bacteria are always associated with mass production of rotifers and may cause unexpected mortalities or suppressed growth of rotifers. Moreover, since they are used as the first food of larvae, rotifers are often suspected as vectors of potential harmful bacteria to the cultured animals (Dhert *et al*. 2001).

The density of egg carrying rotifers in the feeding trial was less than 30 % throughout the experiment, which is a desirable fertility minimum during the culture of rotifers. The percentage of rotifers with eggs is a useful indicator of the health of rotifer population. A rotifer population with ≥30 % egg percentage is consistent with a healthy culture, while a drop in this percentage to 15-20 % is still acceptable, but the growth of the culture will be reduced. A decrease to <15 % often indicates that conditions in the culture tank are sub-optimal, likely due to feeding rate or low water quality (Delbos *et al*. 2001). Low egg percentages were indeed observed and associated with the high mortalities in tanks fed with algae paste.

The disinfection of rotifers with Pyceze (50 % bronopol) proved very effective in lowering microbial counts but the 6 hour treatment caused high mortalities of the rotifers. A disinfection protocol including 0.1 g/L Pyceze (50 ppm bronopol) for 2 hours was highly effective without killing the rotifers and was considered to be the best treatment protocol for rotifers in the study. A lower bronopol concentration was not tested in this study but it might have been more effective in the long term treatment. The producer, Novartis Animal Health, has also tested the effect of bronopol for rotifer disinfection and found out that the disinfection efficiency increases with the increase in chemical dosages, as well as the rotifer mortalities (Novartis Animal Health 2014).

The brine shrimp disinfection trials showed that the initial densities were mostly maintained in the control and Sanocare ACE groups. Pyceze, however, cannot be used as a sole treatment agent for brine shrimps as proven by the study. It has been observed that bronopol has adverse and potentially lethal effects on brine shrimps, depending on duration and concentration of treatment. Sanocare ACE is needed for water quality and provides good protection from the harmful effects of bronopol. Sanocare ACE also had a significant antimicrobial effect but this effect was greatly enhanced when combined with bronopol. A combination of Sanocare ACE (0.6 g/L) and Pyceze (0.2 g/L, 100 ppm bronopol) for 22 hours was very effective in reducing microbial counts, with a moderate negative density effect (-15 to -20 %) and was therefore regarded the best treatment for brine shrimp disinfection. National Lobster Hatchery (2014) had similar results as in the present study, with 14-22 % mortalities and 150 mg/L as the most effective treatment (87 % reduction in CFU as compared with 95 % in the present study).

All the specific objectives defined for this study were realised with potentially interesting results. The study has successfully tested disinfection protocols that were highly effective with regards to survival and feed quality of rotifers and brine shrimp. It is both easy and essential to disinfect live feed to eliminate any pathogens which could have undesirable effects on the culture of marine fish larvae. The results from this study may prove very useful in improving marine larvae culture in South Africa as it has been suspected that the mortalities observed during the larval state of dusky kob (*Argyrosomus japonicus*) might be the result of high microbial loads in live feed cultures. The results from this study may be regarded to improve the management practices of aquaculture in South Africa in accordance to the objectives specified in the National Aquaculture Strategic Framework for 2013.

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APPENDIX: TABLES AND FIGURES

Table 6: Water quality parameters and rotifer population densities during the rotifer feeding..trial.

Day	Silo Number	Temperature (°C)	Oxygen (ppm)	Salinity (ppt)	Wv (L)	D (NEC)	D(EC)	Total D/ml	Total D (M)	Egg %	AVR D(ml)	IN D (ml)	AVR D (M)	SGR (rotifers/day)	Mean Egg %
1	1A	26.8	20.2	32.5	25	952	192	1144	28600000	17					
1	1B	26.8	17.7	32.6	25	860	232	1092	27300000	21					
1	1C	26.8	21.0	32.6	25	772	164	1006	25150000	16	1081	6,99	27016667	0	18
1	2A	26.7	19.6	32.6	25	1056	254	1092	27300000	23					
1	2B	26.6	24.9	32.6	25	1204	284	1134	28350000	25					
1	2C	26.4	23.6	32.6	25	1112	204	1028	25700000	20	1085	6,99	27116667	0	23
1	3A	26.3	18.5	32.4	25	684	120	1092	27300000	11					
1	3B	26.3	17.0	32.5	25	916	152	1068	26700000	14					
1	3C	26.3	19.2	32.5	25	816	152	1281	32025000	12	1147	7,04	28675000	0	12
2	1A	26.7	20.5	31.1	25	1106	235	1341	33524000	18					
2	1B	26.8	19.8	32.0	25	1134	244	1378	34452000	18					
2	1C	26.6	14.5	31.9	25	1206	274	1480	37004000	19	1400	7,24	34993333	13	18
2	2A	26.4	16.8	32.0	25	1382	231	1513	37816000	15					
2	2B	26.7	16.8	31.8	25	1074	225	1299	32480000	17					
2	2C	26.2	16.7	31.9	25	1454	249	1703	42572000	15	1305	7,32	37622667	16	16
2	3A	25.5	20.7	32.3	25	994	166	1160	29000000	14					
2	3B	25.4	17.3	32.0	25	848	164	1012	25288000	16					
2	3C	25.2	19.9	32.2	25	1334	269	1603	40078000	17	1258	7,14	31455333	5	16
3	1A	27.7	19.6	30.9	25	982	174	1146	28652000	15					
3	1B	28.0	23.0	30.9	25	1220	341	1661	41528000	21					
3	1C	27.6	18.3	30.8	25	1122	168	1290	32248000	13	1366	7,22	34142667	12	16
3	2A	27.2	11.6	31.3	25	836	92	928	23200000	10					
3	2B	27.2	19.0	31.6	25	1008	189	1197	29928000	16					
3	2C	26.9	16.0	30.9	25	1154	247	1401	35032000	18	1175	7,07	29386667	4	14
3	3A	26.5	10.2	31.3	25	776	41	817	20416000	5					
3	3B	26.5	11.6	31.2	25	1024	159	1183	29580000	13					
3	3C	26.4	14.5	31.2	25	1170	111	1281	32016000	9	1093	7,00	27337333	-2	9
4	1A	27.2	16.9	30.7	25	1364	316	1680	41992000	19					
4	1B	27.4	22.4	30.7	25	914	153	1067	26680000	14					
4	1C	27.2	19.2	30.8	25	1346	352	1698	42450000	21	1482	7,30	37042667	16	18
4	2A	26.9	15.0	30.9	25	756	149	905	22620000	16					
4	2B	27.0	15.8	30.9	25	1058	236	1292	32306000	18					
4	2C	25.8	18.3	30.5	25	1520	280	1800	45008000	16	1332	7,19	33311333	10	17
4	3A	26.2	15.7	31.2	25	326	91	617	15428000	15					
4	3B	26.3	15.1	29.2	25	780	64	844	21112000	8					
4	3C	26.2	15.2	30.7	25	872	135	1007	25172000	13	823	6,71	20570667	-17	12
5	1A	27.7	16.4	30.1	25	1504	374	1958	48952000	19					
5	1B	28.1	21.6	30.0	25	1178	335	1513	37816000	22					
5	1C	27.6	15.3	29.9	25	1462	515	1977	49416000	26	1816	7,50	45394667	26	22
5	2A	27.1	16.8	30.4	25	1184	343	1527	38164000	22					
5	2B	27.3	17.7	30.2	25	1324	230	1554	38860000	15					
5	2C	27.1	11.3	29.5	25	1444	231	1675	41876000	14	1585	7,37	39633333	19	17
5	3A	27.3	0,01*	29.8	25	47*	0*	51	1276000	0					
5	3B	26.4	0,01*	29.6	25	330*	35*	385	9620800	11	552	6,31	13804000	-37	9
5	3C	26.5	0,06*	29.8	25	1028	192	1220	30500000	16					

Table 7: Water quality parameters recorded during the rotifer disinfection experiment.

Tank No	Temperature	Oxygen (ppm)	Salinity (ppt)	V (L)
1A: 2hours	26.3	20.3	32.0	6
1B: 2 hours	26.0	23.9	32.1	6
1A: 6 hours	26.2	20.3	32.1	6
1B: 6 hours	26.3	23.8	32.1	6
2A: 2 hours	26.1	25.2	32.0	6
2B: 2 hours	25.9	22.3	32.0	6
2A: 6 hours	26.2	25.3	32.0	6
2B: 6 hours	25.8	22.1	32.1	6
3A: 2 hours	26.2	26.5	32.0	6
3B: 2 hours	26.1	25.8	32.0	6
3A: 6 hours	26.0	26.3	32.0	6
3B: 6 hours	26.2	25.7	32.0	6

Key: V = Water volume

Table 8: Population densities after two- and six hour treatment of rotifers during the disinfection trial.

Duration (hours)	ID	FD	ID	FD	ID	FD	SE	SE
2	2000	1940	2000	1940				
2	1510	1504	2000	1992	2000	1966	0	26
6	2184	2040	2000	1868				
6	1976	1830	2000	1852	2000	1860	0	8
2	1891	1888	2000	1997				
2	2108	2104	2000	1996	2000	1997	0	0
6	1680	796	2000	948				
6	1968	1012	2000	1028	2000	988	0	40
2	1975	1972	2000	1997				
2	1730	1724	2000	1993	2000	1995	0	2
6	1752	720	2000	822				
6	1472	620	2000	842	2000	832	0	10
	1853,8	1512,5	2000	1606,3				

Key:
ID = Initial density (normalised).
FD = Final density (normalised).
SE = Standard error.

Table 9: Water quality parameters recorded during the disinfection trial of brine shrimps.

Tank no	Temperature	Oxygen (ppm)	Salinity (ppt)	V (L)
1A: 10 hours	26.2	21.2	31.8	6
1B: 10 hours	26.0	26.6	31.9	6
1A: 22 hours	26.1	21.1	31.7	6
1B: 22 hours	26.1	26.4	31.8	6
2A: 10 hours	26.22	18.8	31.9	6
2B: 10 hours	25.9	24.7	31.9	6
2A: 22 hours	26.9	18.4	31.6	6
2B: 22 hours	25.8	25.3	31.8	6
3A: 10 hours	26.2	23.3	31.9	6
3B: 10 hours	26.0	23.6	31.9	6
3A: 22 hours	26.2	23.1	31.8	6
3B: 22 hours	26.1	23.0	31.7	6
4A: 10 hours	26.2	22.6	31.9	6
4B: 10 hours	26.0	21.5	31.9	6
4A: 22 hours	26.1	22.6	31.9	6
4B: 22 hours	26.1	22.5	31.8	6
5A: 10 hours	26.2	19.9	31.9	6
5B: 10 hours	26.0	21.1	31.9	6
5A: 22 hours	26.2	21.0	31.9	6
5B: 22 hours	26.0	19.7	31.9	6
6A: 10 hours	26.2	19.7	31.9	6
6B: 10 hours	26.0	18.8	31.9	6
6A: 22 hours	26.1	19.7	31.9	6
6B: 22 hours	26.0	18.5	31.9	6

Table 10: Population densities counted before and after disinfection of brine shrimps during the disinfection trial. The chemicals uded were Sanocare ACE (S) and Pyceze (P). P200 and P400 mean 200 ppm and 400 ppm Pyceze, respectively.

Treatment	Tank	Repl.	Duration (hours)	ID	FD	ID	FD	SE	SE
0	1	A	10	300	308				
0	1	B	10	300	278	300	293	0	15
0	1	A	22	300	296				
0	1	B	22	300	308	300	302	0	6
S	2	A	10	300	226				
S	2	B	10	300	239	300	233	0	7
S	2	A	22	300	290				
S	2	B	22	300	298	300	294	0	4
S+P200	3	A	10	300	211				
S+P200	3	B	10	300	272	300	242	0	31
S+P200	3	A	22	300	246				
S+P200	3	B	22	300	246	300	246	0	0
S+P400	4	A	10	300	240				
S+P400	4	B	10	300	238	300	239	0	1
S+P400	4	A	22	300	280				
S+P400	4	B	22	300	238	300	259	0	21
P200	5	A	10	300	252				
P200	5	B	10	300	200	300	226	0	26
P200	5	A	22	300	100				
P200	5	B	22	300	74	300	87	0	13
P400	6	A	10	300	242				
P400	6	B	10	300	218	300	230	0	12
P400	6	A	22	300	10				
P400	6	B	22	300	12	300	11	0	1

Key:
ID = Initial density.
FD = Final density.
SE = Standard error.

Table 11: Total CFU counted after disinfection of rotifers with Pyceze (50% bronopol) for 2 and 6 hous.

Tank No	Time (Hours)	Treatment (Pyceze)	CFU count	10^3 CFU	Rotifers/µl	Rotifers/µl	CFU/rotifer	CFU/rotifer	SD	SE
1A	2	0	364	364.000	300	300	1.213	1.335	172	122
1B	2	0	437	437.000	300		1.457			
2A	2	50	271	271.000	300	300	903	890	19	13
2B	2	50	263	263.000	300		877			
3A	2	100	245	245.000	300	300	817	713	146	103
3B	2	100	183	183.000	300		610			
1A	6	0	344	344.000	300	300	1.147	998	210	148
1B	6	0	255	255.000	300		850			
2A	6	50	217	217.000	300	300	723	657	94	67
2B	6	50	177	177.000	300		590			
3A	6	100	150	150.000	300	300	500	518	26	18
3B	6	100	161	161.000	300		537			

Key: CFU=Colony forming units.

Table 12: Total CFU counted after 10 and 22 hours of disinfection of brine shrimp with Pyceze (50% bronopol) and Sanocare ACE.

Tank No	Time	Treatment	CFU counts	10^3 CFU	Artemia/µl	Artemia/µl	CFU/artemia	CFU/artemia	SD	SE
1A	10	0	232	232.000	30	30	7.733	7.433	424	300
1B	10	0	214	214.000	30		7.133			
2A	10	600 S	166	166.000	30	30	5.533	3.467	2.923	2.067
2B	10	600 S	42	42.000	30		1.400			
3A	10	200 P+600 S	143	143.000	30	30	4.767	2.483	3.229	2.283
3B	10	200 P+600 S	6	6.000	30		200			
4A	10	400 P+600 S	9	9.000	30	30	300	383	118	83
4B	10	400 P+600 S	14	14.000	30		467			
5A	10	200 P	36	36.000	30	30	1.200	1.017	259	183
5B	10	200 P	25	25.000	30		833			
6A	10	400 P	53	53.000	30	30	1.767	1.167	849	600
6B	10	400 P	17	17.000	30		567			
1A	22	0	71	71.000	30	30	2.367	2.217	212	150
1B	22	0	62	62.000	30		2.067			
2A	22	600 S	27	27.000	30	30	900	1.150	354	250
2B	22	600 S	42	42.000	30		1.400			
3A	22	200 P+600 S	14	14.000	30	30	467	433	47	33
3B	22	200 P+600 S	12	12.000	30		400			
4A	22	400 P+600 S	105	105.000	30	30	3.500	3.250	354	250
4B	22	400 P+600 S	90	90.000	30		3.000			
5A	22	200 P	29	29.000	30	30	967	1.767	1.131	800
5B	22	200 P	77	77.000	30		2.567			
6A	22	400 P	113	113.000	30	30	3.767	3.583	259	183
6B	22	400 P	102	102.000	30		3.400			

Key:
B = Bronopol
S = Sanocare ACE

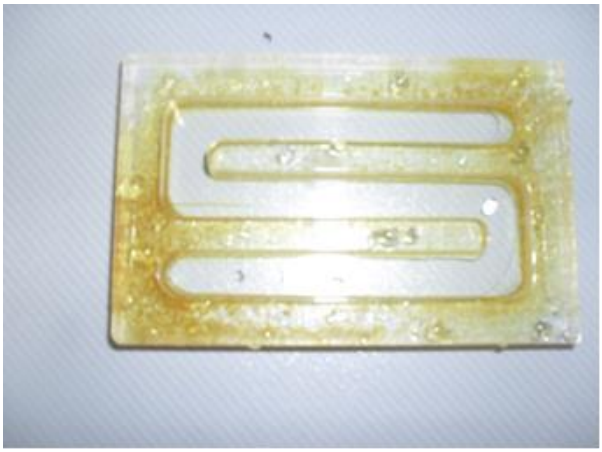

Figure 14: An s-shaped tray used for rotifer- and brine shrimp density determination.

Figure 15: Sponge filters used for trapping suspended particles in the rotifer feeding trial.

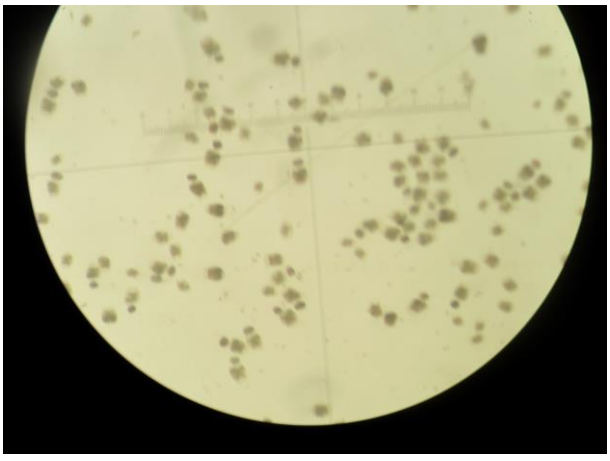

Figure 16: Appearance of rotifers under a 40X magnification using a light microscope during population density counting.

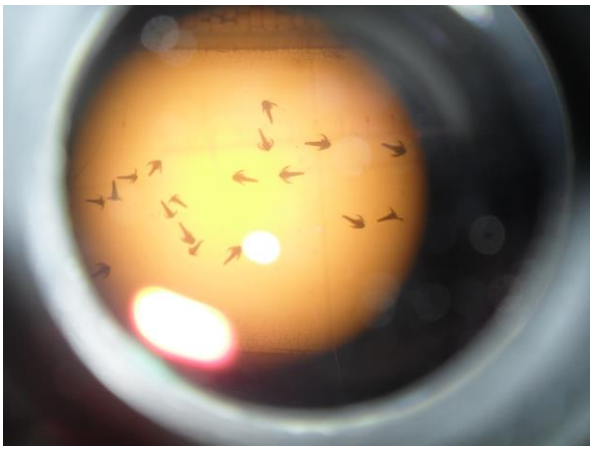

Figure 17: Appearance of brine shrimp under a 40 X magnification in a light microscope used during population density counting.

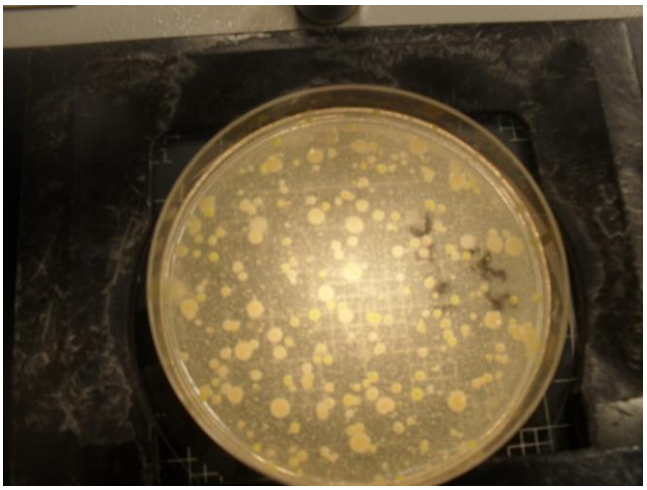

Figure 18: Colonies observed from plates during microbial analyses of rotifers.

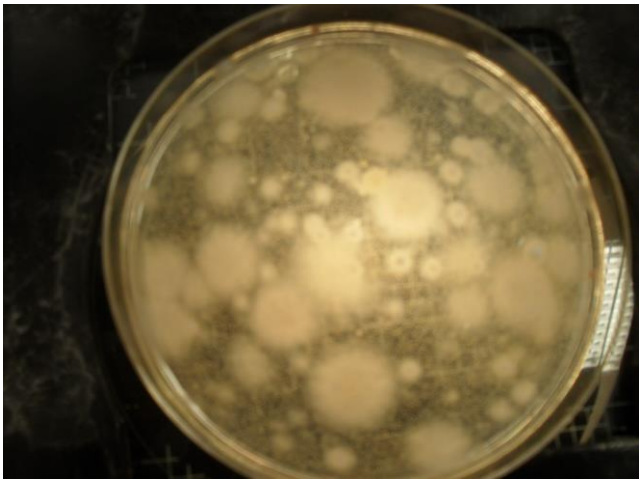

Figure 19: Colonies and fungal growth observed from plates during the microbial analysis of brine shrimp.