Phibro ANIMAL HEALTH CORPORATION TM	Mid-summary Report	Chemical and natural solutions	15.1.2021
Control of adult stage parasitic copepod in fish, Israel			

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Phibro Aqua and the Israeli fish farmers organization joint project

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Introduction

This summary report presents potential solutions, both chemical and natural, to control of copepods parasites in the Israeli aquaculture industry. Copepods are long recognized and their presence in aquaculture cause a threat on fish growth, reproduction and in severe outbreaks, mortality (Johnson et al., 2004). In Israel, on top of the health issue, copepods can cancel of marketing due to the fact that this is crustacean which is a non-kosher animal. the presence of copepod is more evident during the summer and so far, Israeli aquaculturists were dependent on immersion treatments with chemicals against copepods. There were few pesticides which were in continues use; this included Malathion and Dipterex (Dylox, Masoten, Neguvon) and Bromex (Organophosphate) (Paperna, 1996). During the last two years, Israeli authorities had banned the use of these products, following the decision of the European union, due to their harmful environmental impacts. To date, the only available product approved for use in Israel is Dagilin which is highly effective against the intermediate (molting) forms of parasitic copepod but will not be effective against the adult phase. As such, there is no regulatory approved solution in Israel to control the adult stages of copepod parasites in fish which cause the most severe threat. This document is a report that bring up to date its viewers of potential chemical products against parasitic copepods outbreak in the Israeli industry. These products are approved for the use in aquatic animals by the European Union and are likely to be approved by the Israeli authority.

Type of parasitic copepods

There are many different species of copepods however there are three main species that are prevalent in the Israeli industry: *Lernaea spp*., *Ergasilus sp*. and *Argulus spp*.

Lernaea spp.:

The most important parasite in this genus is *Lernaea cyprinacea*, the anchor worm. Although normally associated with carp, they are not host- specific. Other *Lernaea* spp. are encountered with other fish species in both fresh and marine water.

This is a highly adapted crustacean whose head takes the form of an anchor that penetrates the host's skin to form an extremely strong and damaging attachment. The

body of the parasite resembles a worm, with vestigial appendages and paired eggs sacs trailing from the posterior end. Parasitic attachment to the host occurs at stage three, when fertilization occurs. The male dies while the female metamorphoses and penetrates the host tissue (Hossain et al, 2018). The parasite does not survive well at low temperatures and cannot reproduce below 15ºC. Therefore, this parasitic copepod is more distressing in warm areas and especially during the Israeli summer as there is a great abundant of this copepod. The anchor worm actively feeds on the tissues of the host, causing a large granuloma. In the area where the parasites enter the skin, there is often inflammation and hemorrhage. In small fish the damage can be severe and if vital organs are penetrated, mortality will transpire shortly after. Even in larger fish, small numbers of copepods can be harmful and cause spoilage, so the fish's market value is reduced. Affected fish show signs of irritation and exhaustion. Wounds caused by the parasite are prone to secondary infections (Hossain et al., 2018).

Ergasilus* sp.*:

This copepod crustacean is a parasite of many freshwater and marine fish. *Ergasilus* may produce two or three generations a year and the females parasitize the fish during the warmer seasons. *Ergasilus* is a gill parasite, finding its nourishment from the brachial epithelium. The destruction of the gill surface often causes secondary bacterial or mycotic infections and death (Williams & Bunkley-Williams, 2019).

***Argulus* spp.:**

Argulus spp. are large, dorsoventrally flattened crustaceans up to 1 cm long. They are widely distributed and are found on many host species. They are motile and are frequently found on the head of the fish or in sheltered areas behind the fins. Fish-to-fish movement is common, and they can survive for extended periods off the host. Mating takes place on the host; the female leaves the fish to lay eggs on vegetation, which hatch to release actively host-seeking larvae. The parasite feeds by piercing the epidermis with a stylet and digesting skin tissue, causing hemorrhagic ulcers, which are prone to secondary infections. Attachment hooks on the underside of the parasite also can cause mechanical damage. Heavy infestations can cause high mortality, particularly in small fish. Mortalities can also occur due to secondary bacterial

infections. Affected fish show signs of irritation and exhaustion. There is evidence that *Argulus* can transmit virus infection (Steckler & Yanong, 2012).

Objective:

The goal of this project is to identify an effective long-term solution to control adult stage of parasitic copepod in fish in Israel. The proposed solutions must be fully approved for use by the Israeli regulatory authorities and abroad and must be cost-effective.

As such, this project will be focusing on two types of solutions:

1. Testing and registering an existing regulatory approved chemically based immersion product for controlling adult stage parasitic copepods in Israel. Based on the fact that all of these currently approved products might be considered hazardous chemicals and from the familiarity with the regulatory trends of the EU (and in Israel), the chemical solution in the project will be a short-term solution for the Israeli aquaculture industry.
2. Development and registration of a natural in-feed solution for controlling adult stage parasitic copepods in Israel. This will serve as a long-term solution.

Potential chemically based products for treating parasitic adult copepods

In this section, a thorough literature review was conducted in order to find a chemical solution that are approved by the EU for the use in aquatic animals. This primary filter narrowed down this search to five products of which, two products were chosen to be examined in a trial.

Screening for regulatory approved products by the EU:

Avermectins - Product ID:

Treatment	Active ingredients	Class	Mechanism of action
In feed solution	Emamectin benzoate	Macrocyclic lactone	Glutamategated chloride channel activation

This is an in-feed solution that is composed of Emamectin benzoate. Avermectins mode of action is by modulating specific glutamate- and gamma-aminobutyric acid-gated anion channels. The increase of chloride ions leads to hyperpolarization which

disturb the nerve system and lead to paralysis and later to mortality (Torrissen et al., 2013). This chemical solution is effective against all developmental stages, but the full effect can only be determined after two to three weeks. In arthropods, they act through ingestion as stomach poisons, and emamectin benzoate has therefore been developed as a premix for medicated feed (Torrissen et al., 2013). Emamectin benzoate is effective against the Caligidae family and to their entire life cycle. This substance is an in-feed solution for salmonids (Horsberg, 2012). The treatment usually last seven consecutive days and remain effective for about two months before the parasites reappear. For the past decade there are growing records around the globe that some copepods developed resistance to this treatment, so it's effective reduced and there are growing effort to find alternatives (Espedal et al., 2013& Horsberg, 2012). Avermectins will not be examined in this project.

Pyrethroids - product ID:

Treatment	Drug	Active ingredient	Mechanism of action
Bath/Immersion	Pyrethrum	Deltamethrin	Voltage-gated sodium channel modulator

This product is used in immersion and is composed of deltamethrin. Pyrethroids mode of action is to inhibit the function of the voltage gate by disrupting the nerve membrane function, especially by their interaction with sodium (Burridge et al., 2014). system which leads to immobility and numbness and then to mortality. This treatment is effective against all developmental stages. Treatment duration is a batch of about 40 minutes with specific concentration. It takes one to two weeks to evaluate the treatment effectiveness. (Torrissen et al., 2013)**.** It is unlikely that a synthetic form of pyrethroids will have a long-lasting effect on other specie by secondary digestion as it is quickly absorbed. However, it might influence the benthic environment and hence benthic species (Burridge et al., 2014). There are evident that this product lost its effect against sea-lice in Norway as records show that the parasitic sea-lice developed some resistance (Torriseen et al., 2013). Since this is a still approved product in the EU and its effectiveness against parasitic copepods is well approved, a communication with the company that produces this product is underway. If we will

succeed to receive all the necessary approvals, a trial using this product will be promoted.

Hydrogen peroxide

Product ID

Treatment	Drug	Class	Mechanism of action
Bath	Hydrogen peroxide	Oxidizer	Gas embolism

Hydrogen peroxide is a powerful oxidizing liquid formulation made of up to 50% active ingredient complex. Its mode of action is by mechanical paralysis as it preoxidize lipids' and cellular organelle membranes, which interrupt the instigation of proteins and enzymes; this disruption cause mortality (Burridge et al., 2010). This product effectiveness is reduced in cold temperatures (below 10 °C) which is less relevant to this area as the highest parasitic copepods' infection are occurring during the summer. This substance produces quick results and is effective against pre-adult and adult stage parasitic copepods (Torrissen et al., 2013). This is likely to be the least toxic formulation among the examined chemical products. Still, this method is not suitable for the Israeli ponds since field observations showed that in the organic-rich environment of ponds, this substance rapidly decomposes (Burridge et al., 2010). Due to the uncertainty of this product effectiveness in pond environment, this product will not be tested.

Organophosphate (Salmosan) - product ID:

Treatment	Drug	Class	Mechanism of action
Bath	Azamethiphos	Organo-phosphate	Inhibits AChE

Organophosphate had a wide range of usage in various parasitic species in the aquaculture industry. During the 90s, there were growing records of increasing resistance of some parasitic species (Torrissen et al., 2013). Due to its environmental impact some forms of organophosphate were banned, (e.g. Bromex). This product mode of action is by impeding the enzymatic activity of acetylcholinesterase (AChE). This enzyme acts as a hydrolysis agent of the neurotransmitter acetylcholine. Once the hydrolyzation process of AChE into choline and acetic acid is impaired, the neural transmitter is damaged. This results in paralysis and follow by mortality (Torrissen et al., 2013). This substance normally comes in the form of powder that need to be wetted. The drug application is by immersion and the treatment lasts half an hour to an hour. This chemical is effective against pre adult and adult stage copepods; its effectiveness can be evaluated few hours following treatment (Burridge et al., 2010 & Torrissen et al., 2013). Since this is a still approved product in the EU and its effectiveness against parasitic copepods is well approved, a communication with the company that produces this product is underway. If we will succeed to receive all the necessary approvals, a trial using this product will be promoted.

Chitin synthesis inhibitors - product ID:

Treatment	Drug	Class	Mechanism of action
Feed	Teflubenzuron	IGR, Growth inhibitor	Chitin synthesis inhibitor
Feed	Diflubenzuron	IGR, Growth inhibitor	Chitin synthesis inhibitor

Chitin biosynthesis inhibitors (diflubenzuron and teflubenzuron) are also used as in-feed compounds. Their mode of action is to inhibit chitin synthesis of the parasite. Since these parasites are all crustacean and chitin is the building stone of their exoskeleton, inhabiting chitin results in soft shell and growth deficiency. This harm the copepod development and proper exoskeleton formation which leads, eventually,

to death; this takes longer than most insecticides (Torrissen et al., 2013). IGRs prevent the insect from reaching maturity by interfering with the molting process. This prevent from the crustaceans to rich maturation and thus reproduce, so this is a long-lasting solution and it effect all developmental stages (Horsberg, 2012). Mortality usually occurs within three to ten days, depending on the product. Treatment is normally given orally, and this is less preferable by the Israeli aquaculturists. Another problematic issue is that the chemicals not only harm the parasitic copepods but also predaceous insects, arthropods and even fish. Therefore, this product will not be examined in this project.

Communication with the commercial companies

Chemical based products

Following the through products' review and with the understanding of the available products which has high probability to be effective and could be register in Israel, we have narrowed the list to two commercial products. These products are listed in the EU as a treatment for ecto-parasite in fish and are used as bath treatment. So far, the following steps were made:

- Phibro Aqua approached the two "chosen" companies in selling their products to the Israeli market following efficacy and safety trials.
- Phibro Aqua presented the companies, the Israel market, its potential and the solutions that are currently been used by the Israeli industry.
- Phibro Aqua presented the companies the trials plan to evaluate the products as treatments against warm water copepods.
- Phibro Aqua initiated with one of the companies a process of importing samples for R&D trials.
- Parallel to importing the product to Israel, Phibro Aqua with collaboration of the manufacturing company started safety trial which take place in one of the manufacturer R&D sites.
- The second manufacturer would like to complete a risk assessment before sending samples to Israel.

- Phibro Animal Health Corporation regulatory team is working on import permit for the relevant samples.
- Phibro Animal Health Corporation regulatory team is working to evaluate the existing data and the regulatory requirements for each of the two products
- Phibro Aqua and the manufacturer agreed the cost analysis will be conducted following the efficacy trial once we will understand the required dose for applying their product in earthen ponds.

R&D testing and evaluation

As mentioned above, the import permit for the R&D is in process, once it is obtained, the Phibro R&D team will begin to plan and initiate a proper trial to examine the effectiveness of the product.

Natural in-feed solution for control of parasitic copepods:

The proposed natural in-feed solution is saponin based. Saponins are steroid or triterpenoid glycosides, common in a large number of plants and plant products that are in common use in human and animal nutrition. Several biological effects have been ascribed to saponins. Extensive research has been carried out into the membrane-permeabilizing, immunostimulant, hypocholesterolaemic and anticarcinogenic properties of saponins and they have also been found to significantly affect growth, feed intake and reproduction in animals (Francis et al., 2002; Francis et al., 2005). These structurally diverse compounds have also been observed to kill protozoans and molluscs, to be antioxidants and act as antifungal and antiviral agents. *Quillia* contain a number of triterpenoids saponins consisting of glycosides of quillaic acid, some sugars (including glucose, galactose, arabinose, xylose and rhamnose) along with polyphenols (including tannins), calcium oxalate and other minor components. A study that was conducted in Chile and attempted to examine the ability of Quillia saponins against the parasitic copepod Caligidosis, showed promising results. This trial was carried out by BioMar in salmon farm. They used quillia that were derived from the soap bark tree, a Chilean endemic tree (quillay). Researchers added this natural in feed quillia to the feed and show significant reduction to the parasitic burden. The evaluation process leading to the discovery of

the effect of extract quillia began with in-vitro testing of different compounds, which allowed to make a selection according to their prohibitive effect on the development of adult sea louse. Subsequently, the compound was tested in an in vivo test in which the rate of infestation sea lice was reduced over 40%. Finally, they used this product in Atlantic salmon farm in Chile and they succeeded a reduction in the rate of infestation by an average of 43% in tests on Atlantic salmon fish farms. This is a natural and in feed solution that has no environmental harm and once its effectiveness will be evaluated, it might be proposed as a long-term solution for parasitic copepod in the Israeli aquaculture industry.

Communication with the commercial companies

Natural based products

Following literature review of several natural active ingredients and based on previous work that was conducted with saponins against cold water copepod, we have decided to test an in-feed solution based on Quillia saponins. So far, the following steps were made:

- Phibro Aqua approached the manufacturer to interest in developing an in-feed solution to warm water copepods for the Israeli market.
- Phibro Aqua presented the potential of the Israeli market and the solutions which are currently been used by the Israeli industry.
- Phibro Aqua presented to the companies of interest a comprehensive plan to evaluate the products as treatments against warm water copepods.
- Phibro Aqua team obtained approval from the Israeli welfare committee to conduct trials to evaluate the efficacy of Quillia saponins against warm water copepods
- Phibro Animal Health Corporation regulatory team applied for an import permit for samples.
- Phibro Aqua obtained and imported a 25kg bag for R&D work.
- Phibro Aqua R&D team and the manufacturer R&D team is working on the development plan, trials and dosages to be tested based on similar experience they have with the active ingredient.

- Phibro Animal Health Corporation regulatory team is working on building the dossier for registration in case our trials will be successful.

Cost analysis of the various available products

Phibro Aqua and the manufacturer agreed the cost analysis will be conducted following the efficacy trial once we will understand the required inclusion rate.

Natural in-feed solution

Since, 25 kg of the examined natural solution has arrived to Phibro Aqua, the trial examining this substance was promoted. A suitable farm with parasitic copepods was found and the following trial was initiated.

Trial title: Examining two Quillia products with different purity and concentration as a mean to treat *Lernaea* in *Carassius auratus*

Goal: Examine two saponin based products with two different concentrations and purity (data) (Product A & B) as a treatment against *Leranea* in *C. auratus.*

Treatments: three (3) treatments with four (4) replicates each: commercial feed (control), commercial feed with Product A (1g /1kg feed)& commercial feed with Product B (1.5g /1kg feed) add as a coating mix with 2% non-purified soy oil.

Figure1: 12 cages stocked with *C. auratus*

Animals: seven hundred eighty (780) goldfish, *C. auratus* with an average weight of 15g divided in 12 cages of 1.3 m^3 (65 animals/cage) .

Duration: the trial will take place for 45 days.

Feed preparation:

- Coat 4kg of commercial feed with 6g of QD100 with 2% of soy oil
- Coat 4kg of commercial feed with 4g of UD100 with 2% of soy oil
- To ensure homogenous distribution, soy oil and the additive will be mixed thoroughly and only then feed will be added

Experimental design:

- All animals should be with an average weight of 15 .
- Animals will be randomly divided to 12 experimental tanks (65 fish/tank) .
- Each cage will be marked prior to feeding in order to avoid mistakes .
- Feeding will be given according to a commercial feeding table.
- At T0, 10 fish from each cage will randomly collected and severity of parasite infection will be measured
- At T15, 5 fish from each tank will randomly collected and severity of parasite infection will be
- At T30, 5 fish from each tank will randomly collected and severity of parasite infection will be
- At T45, trial termination, 10 fish from each tank will randomly collected and severity of parasite infection will be
- If needed, additional of 14 days will be added to the experiment in order to determine improvement .

Analysis:

- The severity of the parasite infection will be measured according to phibro's protocol
- In order to compare between the treatments, one-way ANOVA tests will be performed using SPSS version 26.

Procedure:

Trial stocking took place on the 15th of October. Fish from a large stock were used for this trial. Fish with *Leranea* were kept aside for the stocking. Following stocking fish were taken to Maayan Tzvi for contamination assessment. Even though there was an attempt to stock infected fish, contamination level was very low, probably due to the

season (fall) as the water are less warm and the prevalence of parasitic copepod is mild

Table 1: contamination level in stocked fish

Fish Number	Number of Parasites	Severity
1	0	Non
2	0	Non
3	0	Non
4	0	Non
5	0	Non
6	0	Non
7	0	Non
8	1	Very low
9	0	Non
10	0	Non
1	0	Non
12	0	Non
13	1	Very low
14	0	Non
15	0	Non
16	0	Non
17	0	Non
18	0	Non
19	0	Non
20	0	Non

Following the stocking, there was an additional effort to contaminate the fish by adding to each cage five fish with at least two mature *Leranea*. Two weeks later, the contamination remains the same and due to that, the trial was terminated and will resume during summer. Other location with infected fish were not found elsewhere.

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