Sustainable aquaculture requires environmental-friendly treatment strategies for fish diseases

Article in Reviews in Aquaculture · June 2019
DOI: 10.1111/raq.12365

CITATIONS
0

READS
509

6 authors, including:

Thora Lieke
7 PUBLICATIONS 26 CITATIONS

Seyed Hossein Hoseinifar
Gorgan University of Agricultural Sciences and Natural Resources
200 PUBLICATIONS 2,448 CITATIONS

Thomas Meinelt
Leibniz-Institute of Freshwater Ecology and Inland Fisheries
96 PUBLICATIONS 1,597 CITATIONS

Bo Pan
145 PUBLICATIONS 4,100 CITATIONS

Some of the authors of this publication are also working on these related projects:

- The effect of short-term starvation and re-feeding on some physiological and biochemical parameters in juveniles sturgeon View project
- Fisheries induced evolution - experimental approach View project
Sustainable aquaculture requires environmental-friendly treatment strategies for fish diseases

Thora Lieke¹,², Thomas Meinelt¹, Seyed Hossein Hoseinifar³, Bo Pan⁴, David L. Straus⁵ and Christian E. W. Steinberg²,⁴

¹ Department of Ecophysiology and Aquaculture, Leibniz Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany
² Faculty of Life Sciences, Freshwater and Stress Ecology, Humboldt University of Berlin, Berlin, Germany
³ Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
⁴ Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, Kunming, China
⁵ U.S. Department of Agriculture, Agricultural Research Service, Harry K. Dupree Stuttgart National Aquaculture Research Center, Stuttgart, AR, USA

Correspondence
Thora Lieke, Leibniz Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12587 Berlin, Germany. Email: lieke@igb-berlin.de

Received 24 December 2018; accepted 5 June 2019.

Abstract
Many classical therapeutants are going to be banned in Europe, and an urgent need for alternatives is emerging. This issue can be exemplified by one major parasitic disease in aquaculture and ornamental fish breeding: velvet disease. This disease, caused by dinoflagellates of the genera Amyloodinium and Piscinoodinium, is an important infection affecting cultured freshwater and marine ornamental and food fish, and consistently causes great financial loss to the associated industries. Therapeutants available contain copper, malachite green, or methylene blue, and which can be toxic to non-target organisms in the surrounding environment. As a result, these chemicals are banned for use by the aquaculture industry in several countries, and a prohibition for commercial ornamental fishkeeping is likely to follow in most countries. Increasing development of resistance to therapeutants, and growing public awareness for animal welfare and environmental protection, have prompted research in the areas of alternative treatment options and immunostimulants. Hydrogen peroxide and peracetic acid are possible ‘green’ therapeutants which do not contribute residues to the environment. Natural feed supplements such as pre- and probiotics can increase animal welfare and prevent stress and/or infections. Humic substances are another promising, natural immunostimulants which will be considered in depth. The aim of this review is to provide an overview of risks and benefits of current treatment options and new approaches to replace harmful therapeutants and minimize the number of toxic residues discharged into the environment. Treatments will be discussed on various parasitic infections and focus, where available, on Amyloodinium and Piscinoodinium.

Key words: biotics, copper sulphate, disease prevention, humic substances, immunostimulants, Oodinium.

Introduction
Finfish are hosts to a plethora of both ecto- and endoparasites. These parasites are part of every ecosystem and they generally have limited effects on the fitness of healthy fish. However, parasites can become a problem under stressful conditions which can occur when fish are reared in captivity (e.g., public aquariums, the ornamental fish trade and aquaculture). In aquaculture, up to 50% of production loss is caused by diseases (Assefa & Abunna 2018). High stocking densities and poor water quality present optimal conditions for the infestation and reproduction of parasites, leading quickly to pathogenic levels. Furthermore, spreading of infectious pathogens is supported by the inevitable transport of fish and equipment (Subasinghe et al. 2001). Parasitic infections can spread not only inside a system but...
also to adjacent culture units via aerosol transfer (Paperna 1980). These anthropogenic stressors can lead to acute and chronic stress, and the need to cope with the allostatic load requires (energetic) metabolic reorganization, subsequently decreasing growth performance, depressing immune system functions and leaving fish even more vulnerable to infections (Bly et al. 1997; Tort 2011). For example, catfish exposed to 30-min low-water stress had about 20% mortality when challenged with *Edwardsiella ictaluri* compared to control fish (Small & Bilodeau 2005). This review focuses on velvet disease, a major disease threat in warm water culture, as a foundation to review the transition to emerging treatment approaches. Information on this disease is often distributed to different databases, paper, and books of research experts. In the first part we summarize information on the different parasites, life cycles, and misinterpretations due to outdated taxonomical terms.

Current treatment options such as triphenylmethane dyes, copper, acriflavine and formalin, as well as their effects on different life stages, are considered. Risks associated with many of these ‘traditional’ chemical therapeutics to fish, to the environment and to consumers have led to bans for their use in the aquaculture industry of many countries. The search for alternative environmentally friendly approaches for diseases has increased during the last decade and shall be considered in detail with emphasis on velvet disease where available.

Furthermore, we reviewed recent international literature, as well as ‘grey literature’ and included results from our own research. Application of hydrogen peroxide (H$_2$O$_2$) and peracetic acid (PAA) as disinfectants to reduce the pathogenic load will be discussed. Supplementing food with live cultures of microorganisms and their immunostimulatory effects have been the subject of many studies in human and fish. We will discuss the different types of “biotics” including their interaction and different modes of action. Finally, supplementation of food and culture water with different plant extracts and humic substances is presented. These supplements are used to increase of overall and immune-related health of fish and thereby stimulation of host-specific defense mechanisms against pathogens. We discuss the idea of whether or not the stimulation of the defense system increases overall protection and helps to prevent velvet disease as well as other pathogen-induced diseases.

**Velvet disease is a risk to both freshwater and marine finfish**

Velvet disease (Noga 2011), also known as Rust or Gold Dust disease, is caused by dinoflagellates of the genera *Amyloodinium* (Brown 1931; Brown & Hovasse 1946) and *Piscinoodinium* (Schäperclaus 1954; Lom 1981) and is a major threat in warm water fish culture facilities. It also poses a threat in coolwater fish culture facilities, although white spot disease, caused by *Ichthyophthirius multifilis* in freshwater and *Cryptocaryon irritans* in marine environments and salmon lice (*Lepeophtheirus salmonis*), and species of the genus *Myxobolus* are more more of a threat. The name ‘Oodinium’, which can be found in old publications, is still used by ichthyopathologists and aquarium hobbyists but does not distinguish between the two genera (Lom & Dyková 1992). Taxonomically, the genus *Oodinium* Chatton, 1912, still exists and contains six species; however, their exact systematic position is unclear (Guiry & Guiry 2018). These species are ectoparasitic dinoflagellates of marine invertebrates. Species of the genus *Crepidoodinium* are also ectoparasites to marine fish but do not cause velvet disease. Figure 1 shows the systematic positions and relations of the different genera.

*Piscinoodinium pillulare* (Figure 2a) and *P. limneticum* are important pathogens of both, tropical and temperate freshwater fishes, while *Amyloodinium ocellatum* (Figure 2b) is the marine analogue and is one of the most important parasites infesting warm water marine or brackish water fishes (Noga 2012). These two dinoflagellate...
genera are morphologically very similar and range in size from 80 to 150 μm. However, *Piscinoodinium* obtains essential parts of its nutrition from photosynthesis, although light is not crucial for survival, while *Amyloodinium* lacks chloroplasts (Hoffman 1999; Abowei et al. 2011; Noga 2012). The life cycle of both genera is controlled by temperature and consists of three stages. First, free-swimming dinospores (also called gymnospores; Lom and Dyková 1992) infect the host and penetrate the epithelium (typically skin or gills) with an extension called a ‘rhizoid’. As Figure 2 indicates, the extension of *Piscinoodinium* is nail-shaped (Abowei et al. 2011), while that of *Amyloodinium* is root-shaped (Lom & Lawler 1973; Cheung et al. 1981). Furthermore, *Amyloodinium* has a special tentacle-like movable organ called a stomopode, which *Piscinoodinium* lacks. The function may be food ingestion and injection of lytic bodies into the host cells (Guillou & Not 2018).

After attaching to the host, dinospores transform into trophonts which feed on the host’s cells. Finally, the parasites detach and transform into tomonts. This encapsulated form is the reproductive stage, and a single tomont produces as many as 256 new dinospores that are ready to infect a new host. Tomont division occurs between 16 and 30°C (Saraiva et al. 2011). At 25°C and under ideal reproduction conditions (e.g. salinity, host density), the entire life cycle (Figure 3) takes about 4 to 5 days.

The high pathogenicity of these organisms is caused not only by extensive structural damage to the epithelium by the rhizoid itself but also by histopathological changes such as degeneration and necrosis in cells adjacent to the attachment site. These changes are possibly induced by toxic substances or irritants produced by the parasites (Lom & Lawler 1973; Paperna 1980). Under such conditions, the host is very vulnerable to osmoregulatory impairment and secondary infections by other pathogens such as bacteria or fungi (Woo & Buchmann 2012).

### Diagnosis, current treatments, and related risks
Velvet disease is highly contagious and considering its rapid lifecycle, it is imperative to diagnose and treat as early as possible. Visible symptoms of an infestation in fish are...
flashing, rapid gill movement, loss of appetite (and consequently loss of weight), clamped fins and lethargy. The key symptom is a velvet film or ‘dusting’ on the skin ranging in colour from gold to brown to green. In an early stage of infestation, symptoms may be weakly pronounced, if they occur at all. Analyzing smears of mucus and skin or gills under the microscope can help to detect dinospores at an early stage of infestation. Since dinospores detach after host death, fish have to be diagnosed while still living or directly after death (Woo & Buchmann 2012). Levy et al. (2007) developed a sensitive and specific PCR assay to detect *Amyloodinium* using ribosomal DNA. They were able to detect a single cell from each of the life cycle stages. An even more sensitive and specific approach is a loop-mediated isothermal amplification (LAMP) assay developed by Picón-Camacho et al. (2013) to detect all life stages of *Amyloodinium*. Using an enzyme-linked immunosorbent assay, Smith et al. (1992) showed the production of antibodies against *Amyloodinium* after infestation; this might also be used to monitor pathogens. All these methods, however, require specific laboratory equipment as well as qualified personnel to perform the assays. Therefore, while they may have great importance for research purposes, microscopy is still the standard diagnostic procedure for practical application in aquaculture facilities.

The infection is usually far advanced before being diagnosed, and by then, fish are already in a weakened condition. At this point, treatment of the disease is often difficult. Prolonged immersion in common, non-iodized salt at about 5 g L\(^{-1}\) (5 ppt or parts per thousand) is an effective treatment against *Piscinooodinium* infestations as the trophonts dislodge (Puello-Cruz et al. 2010). Conversely, short periods of hyposalinity (0 g L\(^{-1}\)) dislodge *Amyloodinium* trophonts from the skin and gills (Kingsford & Lawler 1975; Lawler 1977). After either treatment, fish need to be transferred to a clean environment to prevent a new infestation, and the treatment has to be repeated several times with periods of a few days recovery between treatments to exclude recurrence. Multiple treatments are essential for an effective regimen because the life cycles of individual organisms are not synchronized; therefore, the parasite may be present in several stages concurrently. Immersion treatments may be employed on a small-scale, but are not logistically practical for public aquaria, large professional aquaculture operations or wholesale of ornamental finfish. In addition, not all fish can tolerate the osmotic changes equally well and immersion treatment, as well as the necessary transfer of fish, adds to increased stress levels.

Commercially available therapeutics for velvet disease contain metals (mostly copper), triphenylmethane dyes, acriflavine, or formalin. These chemicals are effective at killing parasites, but are also harmful to non-target organisms, in particular plants and invertebrates such as crustaceans and gastropods (Hodson et al. 1979; Flemming & Trevors 1989). Not only the basic substance, but residues and derivatives of these compounds can be unpredictably toxic to the environment and pose a high risk to fish, particularly when already weakened by infection (Alderman & Clifton-Hadley 1993; Jung et al. 2001; Srivastava et al. 2004).

Copper sulphate (CuSO\(_4\)) is the most commonly used therapeutic to treat *Piscinooodinium* and *Amyloodinium* infestations. Concentrations of 0.5 to 10 mg L\(^{-1}\) CuSO\(_4\) were lethal to sporulating tomonts and dinospores of *Amyloodinium* but did not interrupt the division process of the encapsulated tomont (Paperna 1984). Exposure to 1 mg L\(^{-1}\) CuSO\(_4\) for 12 to 24 h reduced the reproductive success of tomonts, but dinospores, which could cause reinfection, were still produced. Ideal treatment strategies would either use concentrations high enough to ensure complete inhibition of the encapsulated tomont or continuous exposure with low concentrations to kill dinospores; this could be harmful or cause stress to fish. Continuous treatment with a desired therapeutic concentration of 0.15 to 0.2 mg L\(^{-1}\) free copper ion for 2 to 3 weeks helped to eliminate infection with *Amyloodinium* in marine systems (Francis-Floyd & Floyd 2011). Concentrations need to be monitored at least once a day as the free copper ion does not stay in solution for long and multiple treatments are often necessary. Problems occur, however, as copper is highly toxic to invertebrates and algae, even at low concentrations (Francis-Floyd & Floyd 2011; Noga 2012). For extremely sensitive fish, copper is toxic at concentrations close to those required for treatment (Cardeilhac & Whitaker 1988). A concentration of 10 \(\mu\)M CuSO\(_4\) (1.6 mg L\(^{-1}\) CuSO\(_4\)) is used to induce leucocyte recruitment in zebrafish larvae (d’Alencón et al. 2010).

Depending on different factors such as alkalinity, organic matter content, as well as adsorption to surfaces, copper can quickly precipitate which reduces the amount of bioavailable copper (Smith et al. 2015). Furthermore, the concentration of specific ions (e.g., Ca\(^{2+}\), Mg\(^{2+}\), Na\(^+\), H\(^+\)) influence the active uptake of copper by Na\(^+\) transporters as they compete for the binding sides (Niyogi & Wood 2004). In freshwater ponds, risk of acute toxicity can be reduced if the concentration does not exceed 1% of the total alkalinity (Boyd & Massaut 1999). McNevin and Boyd (2004) reported that 99% of the administered copper is bound to pond sediments. To decrease the likelihood of copper entrance into the effluents, water should not be discharged for at least 72 hours. When copper is used in the marine environment, the concentration must be monitored frequently because the free copper ion is unstable (Francis-Floyd & Floyd 2011; Noga 2012). Different ion configurations of copper might have different specific toxicities; however, the evidence is contradictory whether Cu\(^{2+}\) or the...
reduced form Cu\(^+\) is more toxic (Fargasová 1998; Mathews et al. 2015). Chelated, or complexed, forms of copper are sometimes recommended as they reportedly reduce the toxicity to fish. However, the bioavailable concentration is also reduced, which limits the effectiveness against pathogens (Keith 1981). While efforts are being made to obtain United States (U.S.) Food and Drug Administration (FDA)-approval for the use of copper sulphate as a therapeutic to treat freshwater fish diseases in ponds and hatcheries (Straus et al. 2016), it is not approved. Likewise, environmental concerns in the European Union (EU) prohibit the use of copper compounds.

The triphenylmethane dyes include malachite green, methylene blue and crystal (gentian) violet. These have been used since the mid-1930s against a plethora of pathogens in fish culture. They are fungicides (especially effective against Saprolegnia spp.), antiseptics and are effective treatments against internal and external parasites (Sudova et al. 2007; Andersen et al. 2009). None of the tested concentrations of malachite green (0.1–100 μg L\(^{-1}\)) had a lethal effect on Amyloodinium, although concentrations of 0.5 μg L\(^{-1}\) suppressed sporulation (Paperna 1984). However, the compounds themselves, as well as their metabolites are toxic and carcinogenic. Malachite green and methylene blue are toxic to channel catfish (Ictalurus punctatus) and rainbow trout (Oncorhynchus mykiss) at concentrations of 0.2 and 16 μg L\(^{-1}\), respectively (Willford 1966). Furthermore, current studies showed malachite green and its reduced form, leucomalachite green, to be in vivo mutagens (Mitrowska et al. 2005, 2008; Culp et al. 2006). The same applies to crystal violet and brilliant green (Thomas & MacPhee 1984; Andersen et al. 2009), and highly concentrated methylene blue has been linked to neurotoxicity and encephalopathy (Sweet & Standiford 2007; Patel et al. 2012). These substances persist in tissue for an extended period of time and therefore pose a risk not only to the fish but also to human health when fish are consumed. These chemicals are labeled as harmful to human health and the environment, and also as toxic by EU regulation (EG1272 2008) 1272/2008 (CLP); they are prohibited for use in aquaculture within the EU. Malachite green and crystal violet are also banned to use on fish intended for human consumption in the US and Canada (Culp & Beland 1996; Srivastava et al. 2004; Andersen et al. 2009).

Acriflavine (acriflavinium chloride) is used to treat and prevent external infections in ornamental fish and has similar antimicrobial and antifungal properties as the triphenylmethane dyes (Plakas et al. 1999). Paperna (1984) reported that total sporulation of Amyloodinium was only suppressed on incubation with 6 μg L\(^{-1}\) acriflavine. This concentration is in the range of the 48 h-LC\(_{50}\) value (6.8 μg L\(^{-1}\)) determined for channel catfish by Clemens and Sneed (1958), while Willford (1966) determined the LC\(_{50}\) value as 33.2 μg L\(^{-1}\). This discrepancy can be caused by different strains of a given fish species as well as differences in the condition of fish, water chemistry or compound quality used for testing. It shows, however, that toxicity can vary massively even in laboratory studies, and application in tanks or rearing facilities can be challenging. Furthermore, Van Duijn (1967) reported on fish sterility after application of acriflavine. This compound binds to DNA and inhibits protein biosynthesis; the mutagenic potential was reported in different species such as Drosophila melanogaster and Chlamydomonas reinhardtii (Tubbs et al. 1964; Xamena et al. 1984; Dorthu et al. 1992). Obstoy et al. (2015) reported DNA damage in cultured human lymphocytes and lung cancer cells after exposure to 0.025% acriflavine. In summary, acriflavine is genotoxic and is banned for use in aquaculture in the EU (Penninks et al. 2017).

As mentioned previously, formalin is also used as a treatment against ectoparasitic infections in cultured fish, including velvet disease. Amyloodinium infestation on the skin and gills of juvenile bullseye puffers (Sphoeroides annulatus) was significantly decreased after treatment with 51 mg L\(^{-1}\) formalin for 1 h or 4 mg L\(^{-1}\) formalin for 7 h (Fajer-Avila et al. 2003). Effectiveness however, is affected by ambient temperature, water quality (i.e., salinity and content of dissolved organic carbon [DOC]), and storage conditions (Abowei et al. 2011). During in vitro studies, trophonts exposed to 25 to 200 mg L\(^{-1}\) formalin retracted their rhizoid and transformed into tomonts within 1 to 3 h (Paperna 1984), explaining the reduced infection load in the studies mentioned above. Complete inhibition of tomont division was observed only after 24 h exposure to 200 mg L\(^{-1}\) at 27–30°C. After 12 h exposure to 200 mg L\(^{-1}\) formalin, cell division was only reduced by about 40% leaving enough motile dinospores for recurrence. Formalin (50 to 150 mg L\(^{-1}\)) has been used successfully to reduce infections with Saprolegnia parasitica in rainbow trout (Gieseker et al. 2006). However, the 96-h-LC\(_{50}\) values of formalin at pH 7 on silver barb (Barbonymus gonionotus Bleeker), common carp (Cyprinus carpio L) and snakehead (Channa striatus Flower) were 75, 107 and 150 mg L\(^{-1}\), respectively (Chinabut et al. 1988), which is in the range of therapeutic concentration. The parent compound of formalin, formaldehyde, is classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (Group 1) (Cogliano et al. 2005; Bianchi et al. 2007). It is currently approved for specific uses in aquaculture in the US, but not in the EU.

Noga (2011) cites a personal communication mentioning chloroquine diphosphate as a treatment against Amyloodinium. Treating false percula clownfish (Amphiprion ocellaris) with 5 to 10 mg L\(^{-1}\) chloroquine diphosphate for 10 days killed dinospores when they were exiting the cyst. Oral administration of chloroquine was used in cultured
red drum (*Sciaenops ocellata*) as a chemotherapeutic agent against amyloodinosis (Lewis et al. 1988). As chloroquine is used as an anti-malaria drug for humans, it is expensive. Furthermore, it is highly toxic to invertebrates, especially echinoderms and corals, and not approved for fish intended for human consumption. In *in vitro* studies using the fish cell line PLHC-1 derived from topminnow (*Poeciliopsis lucida*) showed reduced lysosomal function, increased metallothionein and glucose-6P-dehydrogenase levels as well as increased activity of succinate dehydrogenase (Zurita et al. 2005).

Another major problem of many therapeutants used against pathogens in fish is the development of resistance. Resistance can often be attributed to misuse or over use of precautionary treatments to prevent infections, or application of low doses for treatments that only kill the weak pathogens. Since therapeutants are applied to the water body, residues are being discharged into the natural environment. This dilution exerts a selection pressure and increases development of resistance even further, which not only endangers fish and environment but also leads to human safety concerns, including human pathogen resistance and foodborne transmission of antimicrobial- and multi-drug resistant microorganisms (White et al. 2002; Hur et al. 2012). Multi-drug resistance (resistance to more than two classes of antimicrobial agents) has been found for example in *Yersinia ruckeri*, *Aeromonas salmonicida* and *Piscirickettsia salmonis* (Balta et al. 2010; Kim et al. 2011).

Furthermore, resistance genes of important fish pathogen species have been found to be commonly located on mobile genetic elements such as the transferable plasmid IncA/C and Class 1 integrons, allowing easy transmission of resistances by horizontal gene transfer, even between different bacterial species (Miller & Harbottle 2018). In contrast to the resistance of bacteria to antimicrobial substances, comparably little is known about the resistance of parasites against chemical treatments. Most occurrences of the resistance of parasites usually originate from human or veterinary medicine. Ectoparasites of fish can be treated with antiparasitic compounds which are routinely used in other higher vertebrates like birds and mammals. Livestock intensification has led to an increased reliance on the use of antiparasitic compounds to control parasites.

In Norway, Scotland and other countries in recent years, the patterns of sea lice (*Lepeophtheirus salmonis*) treatment have changed as the resistance to traditional treatments has increased (Sevatal et al. 2005; Costello 2006; Lees et al. 2008; Jones et al. 2013). The emergence of resistant parasites has limited the choice of effective compounds with a resulting adverse effect on animal welfare and loss in animal productivity (Taylor 1999). Resistance of parasites was further described by Roth et al. (1993), Burka et al. (1997) and Athanassopoulou et al. (2009) against organophosphates, commonly used against parasitic crustaceans and monogeneans. The use of drugs like trichlorfon or praziquantel in bath treatments for ectoparasites also has the disadvantage of resistance development (Umeda et al. 2006). Reverter et al. (2014) report the resistance of platyhelmintes to praziquantel. There is limited available knowledge about any resistances of *Oodinium* against antiparasitic compounds, but the development of resistance must be expected.

**Alternative treatment strategies**

The downside of many ‘traditional’ chemical therapeutants as well as the lack of substances allowed in aquaculture, require new strategies that have led to research focusing on alternative therapeutants. In general, there are two different approaches: 1) treatment of the culture water and the pathogen to reduce the overall load, and 2) treatment of the fish to make them less susceptible against infections by increasing the general stress resistance or by activating defense systems (stimulation of immune response) that protect against the pathogens.

**Hydrogen peroxide and peracetic acid as water disinfectants**

Research on alternative therapeutants for the treatment of velvet disease is rare and the few existing studies evaluate the effects on *Amyloodinium*. Montgomery-Brock et al. (2001) studied the effect of H₂O₂ on trophonts of *Amyloodinium* in cultured Pacific threadfin (*Polydactylus sexfilis*). Application of 75 and 150 mg L⁻¹ H₂O₂ for 30 min resulted in a reduced number of parasites. However, treatment with 300 mg L⁻¹ H₂O₂ resulted in mortality of exposed fish. Treating European seabass (*Dicentrarchus labrax*) with 100 and 200 mg L⁻¹ H₂O₂ for 30 min significantly decreased the *Amyloodinium* load on gills (Seoud et al. 2017).

An alternative to using H₂O₂ is the application of PAA (also known as peroxyacetic acid or acetylhydrogen peroxide), which is a stabilized mixture of acetic acid, H₂O₂, and water. It is used as a disinfectant for agriculture, food processing, and medical and veterinary facilities (Malchesky 1993; EPA 2005). More recently, PAA has been used in wastewater treatment, commercial laundries and in aquaculture in several countries (Straus et al. 2017). Peracetic acid has proven to be an effective treatment against various aquatic pathogens including fungi and bacteria (Jussila et al. 2011; Marchand et al. 2012). Straus et al. (2012) found that 2.5 mg L⁻¹ PAA was effective at controlling saprolegniasis on channel catfish eggs, whereas Mitchell et al. (2009) reported that 250 mg L⁻¹ H₂O₂ was the optimal treatment in an earlier study with *Saprolegnia* in the
same system. Furthermore, PAA can be used as a treatment against the fish parasite *Ichthyophthirius multifiliis*, which causes Ich, or white spot disease in freshwater fish. This ciliated protozoan has a triphasic life cycle similar to *Piscinoodinium* and *Amyloodinium*. Meinelt et al. (2009) reported that different developmental stages of *I. multifiliis* have varying degrees of sensitivity to PAA. Application of 0.6 to 0.9 mg L\(^{-1}\) PAA killed up to 82% of newly released tomonts, but none of the tested concentrations (0.5 to 3.0 mg L\(^{-1}\)) halted theront production of encysted tomonts. Picón Camacho (2010) reported that treatment with 8 to 15 mg L\(^{-1}\) PAA for 1 h was effective at killing all developmental stages, including cysts.

Similar to H\(_2\)O\(_2\), PAA produces hydroxyl radicals that oxidize enzymes and proteins, and increase the permeability of cell walls by destruction of sulphydryl and sulfur compounds. But is more potent than H\(_2\)O\(_2\) because of its fat solubility; in contrast to the microbial breakdown of H\(_2\)O\(_2\) by catalase activity, PAA is primarily degraded by chemical oxidation (Block 1991, 2001; Kitis 2004). This non-specific mode of action prevents adaptation of microorganisms, therefore preventing the development of resistance. The advantages of H\(_2\)O\(_2\) and PAA over conventional therapeutants become even more apparent when considering environmental impact. While H\(_2\)O\(_2\) degrades to water and oxygen, PAA degrades to water, oxygen and acetic acid, which is quickly metabolized by microorganisms. In contrast to conventional therapeutants, no toxic residues are discharged into the environment. Thus, H\(_2\)O\(_2\) and PAA have great potential to be effective against *Piscinoodinium* and to replace conventional therapeutants. Future research determining effective concentrations to treat the different lifecycle stages and the effects of both substances on the hosts would be extremely valuable.

**Vaccination to enhance the immune system**

As in terrestrial vertebrates, vaccination is a method to protect against infections using the adaptive immune system. Exposition to an immunogen primes the immune system by forming T- and B-cells. If challenged again later, these primed cells recognize specific antigens, starting defense mechanisms and antibody production.

Oral vaccination with antigens was tested, but gave controversial results as antigens are often degraded by the gut biota (Hart et al. 1988; Irie et al. 2005). Most commonly, fish are vaccinated by dip or bath immersion, oral vaccination or by intramuscular or intraperitoneal injection (Sommerset et al. 2005). Immersion is an easy way to vaccinate and can be enhanced by low frequency sonophoresis to increase skin permeability during the bath (Fernandez-Alonso et al. 2001; Zhou et al. 2002). As gills are very sensitive to sonication, intensities to be used have to be evaluated carefully (Cobo et al. 2013; Cobo Labarca et al. 2015). A major problem with oral vaccination is that doses are not distributed equally among all fish but differ based on feeding behavior. Furthermore, the harsh gastric environment often breaks down the antigen, making it ineffective (Embregts & Forlenza 2016). Encapsulation with microalgae, alginate particles, nanoparticles or biofilms for example could help reduce this breakdown (Mutoloki et al. 2015; Embregts et al. 2019). Injecting the fish is the most effective way of protection. Vijji et al. (2013) treated ornamental goldfish with whole cell, extracellular products, outer membrane proteins and biofilm vaccines from *Aeromonas hydrophila* which decreased mortality when the fish were challenged. Furthermore, they supplemented the vaccination with extracts from *Asparagus racenosus* which increased phagocytosis, bactericidal activity of the serum and albumin:globulin ratio compared to the group without immunoadjuvant.

In order to produce vaccination against parasites, those parasites have to be cultivated. In some cases, rearing can be done *in vitro*; *A. ocellatum* can be propagated using G1B cells (Noga 1987; Cobb et al. 1998). Often a cultivation of parasites requires a fish host population making the vaccination expensive and laborious. Recent advances include the development of multivalent vaccines, synthetic peptides, and subunit vaccines that use immunogenic parts (Plant & LaPatra 2011; Dadar et al. 2017). However, injection vaccination requires separate handling of each fish resulting in stress and subsequent feed intake reduction or injuries; fish also have to be of sufficient size (excluding young fingerling) to be eligible for injection vaccination. For more details about the present status of fish vaccination, we recommend the reviews by Muktar et al. (2016) and Assefa and Abunna (2018).

**Immunomodulatory feed supplements as an approach to increase animal welfare**

Chronic stress and infection lead to immune system suppression of the host (Tort 2011; Zanuzzo et al. 2019). As a result, the defense systems are weakened and susceptibility to disease is increased. Subsequently, a treatment against an infestation is inevitable; however, as explained above, many conventional therapeutants can be toxic and cause additional stress which may result in loss of fish. Furthermore, inside the EU, many of the traditional chemical therapeutants are already banned. An alternative approach is to prophylactically stimulate overall fish health and immune-related health to prevent diseases (Dawood et al. 2018).

---

Stimulation of the fish’s immune system and other defense mechanisms would have an effect primarily on the free-swimming dinospores. Increased mucus, for example, can prevent the dinospores from attaching to the skin and inhibiting the pathogen from completing its life cycle. Enzymes and other defense mechanisms in gill and skin epithelial cells could affect dinospores attached to the fish. This mucus immunity will be discussed in later. Research on the effects of immune stimulatory substances against Amyloodinium or Piscinoodinium is scarce or even nonexistent. The following section will, therefore, focus on research with regard to increasing overall health and defense mechanisms with a focus on velvet disease were information is available. However, even studies about defense mechanisms against other pathogens give important information and may be worthwhile avenues to investigate for preventing outbreaks of velvet disease. We recommend the book entitled ‘Aquatic Animal Nutrition’ (Steinberg 2018) that explains how diets define the digestive tract and its microbiota, which in turn influences life history and behavioral traits of the host.

**Vitamins**

A body of evidence exists illustrating the effects of dietary vitamin supplementation on health benefits. Vitamins are widely known to improve growth and survival and stimulate the immune response. Lim et al. (2002) reported that feeding 1,000 to 2,000 mg kg⁻¹ vitamin C increased resistance against osmotic stress in guppy (Poecilia reticulata). Furthermore, vitamin C enhanced resistance to infections in various fishes (Li & Lovell 1985; Navarre & Halver 1989; Hardie et al. 1991) and vitamin E supplements decreased stress caused by the fungicide copper oxychloride in Nile tilapia (Oreochromis niloticus) (Hassan & et al. 2014). As mentioned previously, reduction of stress will make fish less vulnerable to infections. Although supplementing fish feed with vitamins is beneficial, and lack of these substances leads to deficiency symptoms, there is also the risk of over-supplementation. Dietary oversupply (hypervitaminosis) of vitamin C and D leads to decreased growth, elevated levels of alkaline phosphatase and lethargy, and this can cause slow wound repair and depressed immunity (Merchie et al. 1996; Darias et al. 2011). In addition, skeletal abnormalities, jaw malformations, albinism and melanism caused by high concentrations of vitamin C have been reported in olive flounder (Paralichthys olivaceus) and in striped trumpeter (Latris lineata) (Negm et al. 2014; Takeuchi 2014). Overdose by supplementing with high concentrations of vitamins is particularly risky in the case of vitamin A. Dedi et al. (1995) demonstrated that feeding olive flounder with Artemia enriched with more than 40 mg vitamin A palmitate produced a negative effect on growth (body weight and total length) and a high incidence of bone deformities. This shows that despite the beneficial effects on fish, similar to ‘traditional’ therapeutics, vitamin supplementation can have its downsides and needs to be used and monitored carefully.

**Prebiotics, probiotics, postbiotics, parabiotics, symbiotics**

Vitamins are not the only group of dietary supplements that have been studied for their health benefit in aquatic animals. There are numerous available studies on the effect of food-supplementation with nutrients (‘functional feeds’) such as amino acids (Tesser et al. 2005; Peres & oliva-Teles 2008), polyunsaturated fatty acids (Martinez-Rubio et al. 2012, 2013), short chain fatty acids (Hoseinifar et al. 2017b) and different kinds of ‘biotics’ have been evaluated in a plethora of studies (Hoseinifar et al. 2018; Van Doan et al. 2019). The following section will discuss the different kinds of biotics, their effects on the immune status and pathogen resistance of fish and effects against aquatic parasites.

Gibson and Roberfroid (1995) define prebiotics as in the following: ‘[Prebiotics] are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health’. Main targets are either the increase in potentially beneficial bacteria proliferation or counteracting the negative ones (Encarnação 2016). Beneficial bacteria, such as Lactobacillus and Bifidobacterium, hydrolyse the prebiotics which results in production of various bio-active compounds such as short chain fatty acids (scFA), vitamins and peptides. These are capable of increasing fitness over less beneficial or pathogenic bacteria, thereby indirectly improving fish health (Hoseinifar et al. 2015a; Hoseinifar et al. 2017c; Jung-Schroers et al. 2016). Furthermore, prebiotics can directly improve fish health by increasing the immune response (Ringo et al. 2010; Song et al. 2014; Torrecillas et al. 2014). Acting as “risk” signals, β-glucans activate defense mechanisms that are also triggered after cell damage including antigen presenting cells and increased production of Th1 and Th2 cells (Dalmo & Bøgwald 2008). Information on combating challenges by parasites is scarce and often inconsistent. Feeding rainbow trout with 0.2% β-glucan Lauridsen and Buchmann (2010) decreased susceptibility to I. multifiliis while plasma lysozyme activity was increased. Feeding common carp with 3% β-1,3/1,6-glucan increased the relative gene expression of interleukin 1-β; however, susceptibility against I. multifiliis was not significantly altered (Herczeg et al. 2017). Protection against the marine parasitic flatworm Neobenedenia girellae was gained after feeding greater amberjack (Seriola dumerili) with different forms of mannan oligosaccharides (Fernández-Montero et al. 2019). A promising study for
the use of prebiotics against velvet disease was conducted by Buentello et al. (2010); in this research red drum feed was supplemented with several prebiotics at 10 g kg$^{-1}$ which resulted in increased serum lysozyme activity and survival after infection with A. ocellatum. Recently, Guerreiro et al. (2018) gave an overview about currently used prebiotics. They point out the importance of different factors that can influence effects such as fish age and size, environmental factors as temperature and give advises on timing and duration of administration as misuse of prebiotics can also cause adverse effects such as alterations in gut histomorphology.

The term probiotics evolved in the last years of research on functional foods, referring to “soluble factors (products or metabolic byproducts) secreted by live bacteria or released after bacterial lysis” (Aguilar-Toalá et al. 2018). These include short-chain fatty acids (scFAs), enzymes, peptides, cell surface proteins, and vitamins (Tsilingiri & Rescigno 2012; Konstantinov et al. 2013). Although immune response promotion was found, the exact mechanisms of postbiotic modes of action have not yet been fully elucidated (Kareem et al. 2016; Nawaz et al. 2018). Recent studies shed light on the mode of action of prebiotic on host immune response. It has been shown that dietary administration of prebiotics and their fermentation by beneficial communities of gut microbiota drastically increase the levels of scFAs, including acetate, propionate and butyrate (Hoseinifar et al. 2017a). These scFAs are capable of modulating immune response through binding to G protein coupled receptor, GPR43, which is expressed mainly on innate immune response and inflammatory cells (Maslowski & Mackay 2011). However, such scFAs-receptors have not been reported in fish.

The FAO/WHO defined probiotics as ‘live microorganisms which, when administered in adequate amounts, confer health benefits on hosts’ (FAO 2001). This includes host-associated, microorganisms, such as Lactobacillus, Bifidobacterium, Vibrio, and Aeromonas, but also yeast (Saccharomyces cerevisiae and Saccharomyces exiguous) and microalgae (Tetraselmis suecica). Although immunostimulatory effects of probiotics have been analysed in several studies, there are limited studies available about effects of probiotics on resistance against parasites. Going into detail would go beyond the scope of this review; we therefore focus on a few selected examples were bacterial and parasitic challenges have been studied and recommend the reviews from Zorriezhzahra et al. (2016) and Hoseinifar et al. (2018). Bacillus strains were screened for their anti-Vibrio properties and the effective mechanism was cell lysis by disruption of cell membranes (Gao et al. 2017a). Zebrafish (Danio rerio) treated with different strains of Lactobacillus and Bacillus amylyoliquefaciens increased transcription of immune related genes, such as insulin-like growth factor-1 (igf-1), complement component 3a (c3a), and genes of interleukins, tumor necrosis factors, toll-like receptors and lysozyme. At the same time, relative expression of genes involved in oxidative stress such as superoxide dismutase (SOD), glutathione peroxidase 1a (Gpx1a), nitric oxide synthase 2a (NOS2a) and heat shock protein 70 (Hsp70) was decreased (Qin et al. 2018; Lin et al. 2019). In addition, mortality was decreased when challenged with A. hydrophila and S. agalactiae. The few reported trials with dietary pre- and probiotics as therapeutic strategy against Amyloodinium are mixed. Reyes-Becerril et al. (2008) investigated the effects of the dietary intake of live yeast Debaryomyces Hansenii on the immune response of juvenile leopard groupers (Mycteroperca rosacea) and on their resistance to infection by A. ocellatum; only fish with yeast-supplemented diet recovered from the parasitic challenge. The underlying stimulated immune response was based on a significant increase in immunoglobulin M content as well as the activity of superoxide dismutase (Reyes-Becerril et al. 2008). The second attempt we are aware of failed; Li et al. (2005) evaluated the effects of dietary supplementation with brewer’s yeast and nucleotides on juvenile red drum’s resistance to A. ocellatum infection and found that in situ challenge by this parasite caused 100% mortality regardless of dietary treatment within a 48 h period. Feeding rainbow trout for 14 days with 10^9 CFU g$^{-1}$ Aeromonas sobria (GC2) resulted in 100% survival after infection with I. multifiliis (0% in control) while, using 10^10 CFU g$^{-1}$ Brochothrix thermosphacta BA211 did not reduce the mortality (Pieters et al. 2008). As mentioned, I. multifiliis and Amyloodinium/Piscinoodinium have similar live cycles; therefore, A. sobria (GC2) might be a treatment option against velvet disease. However, BA211 significantly increased survival against Aeromonas bestiarum (Pieters et al. 2008). These results show that one probiotic strain can have different effects on different challenges, and that effects of timing, age and species of fish, as well as effect against specific pathogen or parasite has to be evaluated carefully prior to being recommended for application in aquaculture facilities.

Concerns have been raised about the functionality and applicability of live microbes including the viability in the supplementing product, persistence in the gut, horizontal gene transfer from pathogenic bacteria, and enhanced inflammatory responses (Kataria et al. 2009; van Reenen & Dicks 2011; Taverniti & Guglielmetti 2011). Inactivated (non-viable) preparations of probiotics (the so-called para-probiotics) have also shown to be effective immune stimulants (Rampengan et al. 2010). Immunostimulatory effects (respiratory burst activity, increases of plasma lysozyme and myeloperoxidase activity) of heat-inactivated Bacillus amylyoliquefaciens FPTB16 were found in the South Asian carp Gibelion catla (Hamilton, 1822) (Singh et al. 2017). In rainbow trout, Enterobacter sp. was used as effective supplementation against Flavobacterium psychrophilum and inactivated Enterococcus faecalis significantly decreased...
cumulative mortality and frequency of infection with *A. salmonicida* (Rodriguez-Estrada et al. 2013; LaPatra et al. 2014). Application in aquaculture is still limited and so far no studies exist evaluating the potential protective effect against parasites. Choudhury and Kamila (2011) presented a good comprehensive review about current studies and the role of paraprobiotics in modifying responses in fish.

Often a combination of different treatment strategies is more successful. Over the past year, an updated idea was suggested regarding modulation of intestinal microbiota aimed at improved host health, the so-called ‘Synbiotic’ (Gibson & Roberfroid 1995). The idea refers to combined administration of a probiotic bacterium with an optimum prebiotic as substrate. Using mannann oligosaccharide combined with *E. faecalis* decreased the frequency of *A. salmonicida* even more than compared to application of each supplement alone (Rodriguez-Estrada et al. 2013). Feeding angelfish (*Pterophyllum scalare*) with symbiotic (*Pediococcus acidilactici* plus fructooligosaccharide) enriched *Artemia* was more effective in increasing skin mucus lysozyme, protease activity, and immunoglobulin concentration than singular enrichment with probiotics or prebiotics alone (Azimirad et al. 2016). Similar results were found after treating rainbow trout for 8 weeks with galactooligosaccharides (GOS), *P. acidilactici* and the combination (Hoseinifar et al. 2015b); activity of serum lysozyme, respiratory burst, and alternative complement was significantly increased compared to singular enrichment. The highest resistance against *Streptococcus iniae* was observed in the synbiotic group. Several combinations have been tested for their stimulatory potential (Cerezuela et al. 2011; Hoseinifar et al. 2016a; Huynh et al. 2017).

Dietary ‘biotics’ exhibit optima with regard to doses and frequency of application, and can exert adverse effects when applied above their optimum concentration or for too long. Feeding tiger shrimp (*Penaeus monodon*) with 1000 mg kg\(^{-1}\) of the immunostimulant alginic acid for 35 days, Ojerio et al. (2018) found an enhanced resistance against white spot syndrome virus (WSSV), while groups fed higher concentrations had reduced survival rates. Apart from the beneficial effects of the feed supplementation on growth and immune parameters, few studies with real challenges against pathogens or parasites have been conducted yet. Molecular studies are also required to assess the mechanisms behind the stimulatory effects, and further understanding of the pathways of these compounds will be a crucial step towards their broad application in aquaculture and the replacement of antibiotics and traditional therapeutants.

**Plant secondary metabolites**

A growing field of research is focusing on evaluating the effect of natural organic matter (NOM), including plant secondary metabolites (PSM), on health benefits. Often inspired by traditional Chinese medicine, a plethora of fish and aquatic invertebrates have been treated with extracts from medicinal plants (i.e., herbal drugs). In addition to the direct promotion of health, such as benefits in growth and food conversion ratio, many PSM isolates increase specific and non-specific immunity, and thereby resistance against stress and various pathogens. One example is the change in the internal cell oxidative status (mitohormesis). Increasing evidence indicates that oxidative burst-released ROS not only cause oxidative stress, but rather may function as signaling molecules that promote health by preventing or delaying a number of chronic diseases, and ultimately extend lifespan (Ristow & Schmeisser 2014).

Apart from increasing the specific growth rate, very low concentrations of anthraquinone extract from rhubarb (*Rheum officinale*) increased the activities of blood lysozyme and resistance against crowding stress or artificial *A. hydrophila* infection in common carp and prevented high temperature stress in giant river prawn (*Macrobrachium rosenbergii*) (Xie et al. 2008; Liu et al. 2010). After treatment with an extract of Siberian ginseng (*Eleutherococcus senticosus*), olive flounder demonstrated increased non-specific immunity and resistance against infection with *Edwardsiella tarda* and *Vibrio anguillarum* (Won et al. 2008). Anusha et al. (2014) reported that extracts from jungle geranium (*Ixora coccinea*) protected goldfish (*Carassius auratus*) against *A. hydrophila* infection. Resveratrol, a phytoalexin found in grapes and several berries can extend lifespan, protect the nervous system, increase cognitive abilities and retard ageing-related histological markers in redtail notho (*Nothobranchius guentheri*) (Yu & Li 2012; Genade & Lang 2013). Although, many studies focus on anti-bacterial effects of PSMs, there is evidence of anti-parasitic and anti-fungal effects as well. Applying extracts of garlic (*Allium sativum*) and Indian almond (*Terminalia catappa*) to rearing water of tilapia fingerlings, Chitmanat et al. (2005) were able to completely remove a *Trichodina* sp. infection After a 2-day treatment. Garlic was also effective at killing theronts (63.5 mg L\(^{-1}\)) and tomocys (570 mg L\(^{-1}\)) of *I. multifilis* (Buchmann et al. 2003). Magnolia (*Magnolia officinalis*), *Euphorbiaeae* (*Euphorbia pisciciana* Steud.), black tyme (*Thymbra specia* L.), oregano (*Origanum onites* L.), and savory (*Satureja tymbra* L.) were used to control *Saprolegnia* sp. (Gormez & Diler 2014; Huang et al. 2015). Extracts from different medicinal herbs, e.g. cinnamon (*Cinnamomum cassia*), golden larch (*Pseudolarix amabilis*), juanilama (*Lippia alba*) applied either as bath or orally, were effective anthelmintics (Ji et al. 2012; Reverter et al. 2014; Soares et al. 2016). These are only a few examples of studies analyzing the effects of medicinal plants and secondary metabolites. Stratev et al. (2018) presented a
comprehensive overview about current treatments using medicinal plants as well as the mechanisms of action.

Humic substances: rediscovered remedy

Major components of Natural Organic Matter (NOM) are humic substances. These ubiquitous, organic compounds arise from the physical, chemical and microbiological transformation (humification) of biomolecules and occur in decaying debris and soil. Up to 95% of dissolved organic matter (DOM) found in aquatic ecosystems are humic substances (Thurman 1985; Haitzer et al. 1998; Steinberg 2003). Humic substances can be divided into three main fractions: humic acids, fulvic acids and humins (Stevenson 1982). These subdivisions are arbitrarily based on the solubility of each fraction in water at different pH levels as well as their molecular weight (Pettit 2004).

Figure 4 shows a schematic structure of a humic acid with various functional groups which are important for interactions with exposed organisms. Most effects of humic substances have been evaluated in terrestrial animals so far. Administration of humic substances as food supplements has been shown to increase vitality traits such as growth and immunity, and reduce diseases in poultry and rats (Yasar et al. 2002; TeraVita 2004, Islam et al. 2005). Vetcicka et al. (2010) tested two different types of humic acids on humoral and cellular immune reactions of mice; evidence of immunomodulating activity was demonstrated through increased phagocytosis by neutrophils and macrophages, as well as stimulated secretion of IL-2 by spleen cells.

Increased vigor, growth, lifespan and increased stress resistance have also been reported after application of 5 to 180 mg L$^{-1}$ DOC of an artificial humic substance (HS 1500) for 21 weeks to culture water of swordtail (Xiphophorus helleri) (Meinelt et al. 2004). In the cladoceran Moina macrocumpa, osmotic stress was reduced in the presence of 10 mg L$^{-1}$ DOC of a natural humic substance from a Brazilian polyhumic coastal lagoon (Suhett et al. 2011); stress resistance was inherited by the succeeding generation, most likely via DNA methylation (Menzel et al. 2011). Supplementing feed with 2 g kg$^{-1}$ of a fulvic acid significantly increased survival of white shrimp (Litopenaeus vannamei) challenged with Vibrio parahaemolyticus (Fierro-Coronado et al. 2018). In this research, TCTP (translationally controlled tumor protein), which is involved in the regulation of protection against pathogens – chemical treatments versus immunostimulants.
of the host defense response against bacterial and viral infections, was significantly increased, while Hsp70 was significantly decreased 72 h after feeding; this indicates a modulatory effect of the fulvic acid on the immune and stress response. Adding 1% of humus extract to the feed of ayu fish (Plecoglossus altivelis) decreased the development of skin lesions during infection with F. psychrophilum (Nakagawa et al. 2009). A direct effect on the pathogen was excluded, as the pathogenic load in the water did not decrease due to the treatment. Supplementing feed of juvenile loach (Paramisgurnus dabyanus (Sauvage)) with 1.5% fulvic acid for 60 days resulted not only in elevated growth performance but in an increase in intestinal protease activity, antioxidant activity, lysozyme activity (LZM), complement 3 (C3) content, immune globulin M (IgM) content, acid phosphatase activity (ACP), and alkaline phosphatase activity (AKP) (Gao et al. 2017b). Intestinal health also increased as shown by elevated abundance of Lactobacillus and decreased amounts of Serratia, Acinetobacter, Aeromonas and Edwardsiella.

Studies using fish eggs and larvae demonstrated that humic substances increased defense against pathogenic fungi (Saprolegnia spp.) and different microorganisms (Aeromonas spp.) (Schreckenbach et al. 1994; Meinelt et al. 2008). Common carp had significantly reduced infection rates when challenged with A. salmonicida subsp. salmonicida after oral application of humic-rich sludge from a recirculating aquaculture system, a synthetic humic acid, and an extract derived from Leonardite (Yamin et al. 2017). Similar results were found by Kodama et al. (2007) after oral administration of humus extracts to carp infected with A. salmonicida. However, research of whether humic substances can be a suitable supplement to reduce stress in fish caused by treatment against, or the infection itself, is still new and only few current studies have examined this topic yet.

Only a few studies have documented the rare phenomenon of bio-toxicity of humic substances (Gau et al. 2000; Chen et al. 2002; Hseu et al. 2008), yet the effect was found in vitro using different cell cultures. Bernacci et al. (1996) examined the in vivo effect by feeding mice a single dose of an aqueous solution containing 4 g L⁻¹ of humic acids (100 mg kg⁻¹ body weight). Orally administered humic acids induced structural and numerical chromosomal abnormalities in intestinal cells. However, the concentration used during these experiments was 10 to 1000 times higher than those that are environmentally realistic.

As mentioned previously, the structure of humic substances is very diverse and different humic substances can therefore provoke contrasting effects. Combating the water mold Saprolegnia parasitica with 20 humic preparations, Meinelt et al. (2007) found that humic preparations with high aromaticity which contain a high number of organic radicals were the most efficient in reducing fungal growth and caused sporangia formation. Steinberg et al. (2007) identified that those humic substances effective with Saprolegnia also triggered longevity in the nematode Caenorhabditis elegans. On the other hand, humic substances with high shares of aliphatic structures, such as carbohydrates, tended to support Saprolegnia growth and did not extend lifespan in C. elegans (Steinberg et al. 2007). These examples show that structure matters and structural aspects of humic substances have to be considered and evaluated when applying humic substances as treatments against velvet disease and similar pathogens.

Increased animal welfare, as well as enhanced immune-related and overall health, may help prevent diseases or help fish recover from infections easier. Whether an increase in mechanical and chemical defense mechanisms by application of humic substances helps to decrease susceptibility to Piscinooodinium and Amyloodinium should be evaluated in future research and may provide an alternative to conventional therapeutants. However, when considering humic substances as a treatment or preventative against fish pathogens, one must keep in mind that the very supplements that improve fish health may also promote the growth of dinoflagellates. Low concentrations (6 to 32 μg mL⁻¹) of humic substances exerted a stimulatory effect that was reflected by increased yield, growth rate and ¹⁴C uptake in marine dinoflagellates of the genus Gonyaulax (Prakash & Rashid 1968). At higher concentrations (over 35 μg mL⁻¹), growth rate and yield significantly decreased. Effects of humic substances on hosts and parasites should be monitored and evaluated carefully to ensure proper treatment. Apart from that, humic substances have the potential to reduce susceptibility to infections and pathogens by increasing animal welfare and stimulating host-specific defense mechanisms.

**Defense mechanisms**

The defense system of vertebrates is organized in two levels 1) the innate and 2) the adaptive defense system. Innate protection has a general character and protects against unspecific pathogens without recognition and is a fast acting defense system. The adaptive protection in contrast depends on recognition of distinct molecular structures of the invading organisms and memory development. Response is slow (weeks to month) (Van Muiswinkel 1995). Innate defense mechanisms of fish include epithelial barriers, lysozyme, phagocytic cells, and interferons and are similar to that of mammals and birds. However, due to the intimate contact with their environment, fish have established some special defense mechanisms which are less pronounced in other vertebrates.
Mucosal immunity

Beside skin, there are other mucosal surfaces in fish which have important barrier function (Salinas 2015; Hoseinifar et al. 2019). These barriers are composed of the epithelia and their mucous secretions, is called mucosa-associated lymphoid tissue (MALT). The MALT is presented in gut (gut-associated lymphoid tissue or GALT), gills (gill-associated lymphoid tissue or GIALT), skin (skin-associated lymphoid tissue or SALT) and as recently discovered in nose (nasopharynx-associated lymphoid tissue (NALT)) (Lazado & Caipang 2014). The skin mucus can be considered a first line of defense against attacks of pathogens and parasites. Consequently, fish have developed means to improve the mucosal immunity. Feeding Persian hogweed (Heracleum persicum) powder to common carp, Hoseinifar et al. (2016b) found significantly elevated immunological factors such as immunoglobulins, lysozyme, protease and alternative complement activities in carp mucus but not in serum. Feeding Caspian white fish (Rutilus frisii kutum) and Caspian brown trout (Salmo trutta caspius) with dietary peppermint extracts increased skin mucus defense (protein concentration, alkaline phosphatase and antimicrobial activity) (Adel et al. 2015). Furthermore, immune genes in mucosal tissues were upregulated and resistance against parasites was increased after feeding Greater amberjack (Seriola dumerilii) with cMOS supplemented diets (Fernández-Montero et al. 2019). These studies indicate that nutrition plays a central role in this defense process. Addition of a humic substance to culture water reduced attachment and penetration of the fish skin by pathogens (Schreckenbach et al. 1996). Furthermore, increased mucosal lysozyme activity was found when fish were exposed to a fulvic acid (which is also a humic substance) while serum lysozyme was unaffected (Lieke, et al., in preparation). This indicates that apart from nutrition, other pathways seem to be involved in regulation of mucosal immunity and the mode of action has not been determined and merit further research.

Antimicrobial peptides are natural defense mechanisms against pathogens

Antimicrobial peptides (AMPs), such as histone-like proteins (HLPs) and piscidines, are a diverse group of molecules that are widespread in the animal and plant kingdoms and are important parts of the innate immune system (Schröder 2013). They were discovered when an extract from a soil Bacillus strain was used to protect mice from pneumococci infection (Dubos 1939a, b). More than 5,500 AMPs have been discovered or synthesized to date (Zhao et al. 2013). In animals, AMPs are located in different tissues; especially those exposed to the environment (i.e., epidermis and gills) and are a first line of defense against pathogens. The AMPs consist of up to 100 amino acids and most demonstrate an amphipathic design with clusters of hydrophobic and cationic amino acids (Schulze 2010). The basis of specificity of AMPs arises from different physicochemical properties of the outer membranes. The outer leaflet of the bilayer of bacteria consists of phospholipids with negatively charged headgroups, while that of plants and animals has no net charge (Op den Kamp 1979; Zasloff 2002). The mode of action of AMPs is not completely understood. According to the Shai–Matsuzaki–Huang–model (Matsuzaki 1999; Shai 1999; Yang et al. 2000), accumulation of AMPs at the outer layer is aided by electrostatic interactions, followed by formation of α-helices and insertion of the hydrophobic clusters leading to membrane permeation. Inhibition of target organisms can be different, including the disruption of membrane integrity and arising plasmolysis, inhibition of proteins, DNA and RNA synthesis and interaction with intracellular targets (Bahar & Ren 2013). Because of the physical disruption of the cell membrane, resistance against AMPs will not develop. Due to the lack of net charge and the high content of cholesterol, AMPs do not accumulate at membranes of multicellular organisms (Matsuzaki et al. 1995; Toke 2005), preventing impairment of non-target organisms. Therefore, they have been analysed as a replacement for traditional therapeutants (Hancock & Patrzykat 2002; Finlay & Hancock 2004) and could be a useful tool to prevent velvet disease.

Noga et al. (2002, 2003) reported negative effects of HLP-1, which has high sequence homology to histone H2B, on the infection intensity and propagation of A. acclatum. Addition of 100 μg mL⁻¹ HLP-1, isolated from rainbow trout (Oncorhynchus mykiss) and hybrid striped bass (female Morone chrysops x male M. saxatilis), inhibited young and mature trophonts in vitro. HLP-1 caused delayed or irregular development of the parasites and led to less aggressive feeding behavior and slower monolayer destruction (Noga et al. 2002, 2003). In vitro upregulation of the AMPs results in expression concentrations, especially in skin and gill epithelia, which are in the range of the anti-parasitic concentrations, measured in vitro (Ullal et al. 2008).

Stimulation of these host-specific defensive mechanisms could help protect fish against velvet disease, as parasites are challenged at an early stage of infection and immune system functions attempt to destroy them. Consequently, these host-specific defense mechanisms decrease the chances of parasite propagation, prevent mass infections and prevent excessively weakened health conditions of fish. As humic substances are immunostimulatory, they may induce expression of AMPs, but there is presently no research about this.
Conclusion

Diseases cause significant financial losses to public aquariums, ornamental enthusiasts and aquaculture operations. Few studies exist evaluating effects of environmentally friendly therapeutants to diseases, such as velvet disease, maybe due to its lower infectivity at low temperatures. Household remedies such as saline baths are often used by hobby aquarists but are unsuitable for commercial usage. Commercially available therapeutants are toxic to non-target organisms and can increase stress levels, lower health conditions, and potentially cause death of treated fish. Discarded residues of these therapeutants can be harmful to the environment or lead to the development of resistance in microorganisms, which can pose a subsequent risk to human safety as well. Especially inside the EU regulations are strict and EU aquaculture is renowned for its high quality sustainability and consumer protection standards. These include regulations of environmental impact assessment, water use, food and safety hygiene, animal welfare, and animal health. The European Commission is helping and demanding to increase a sustainable aquaculture that is safe but also competitive with other countries. This shows the high demand of replacing chemical therapeutants not only by the government but by end consumers as well, and has fueled research approaches on alternative strategies such as disinfection of water, immunostimulatory feed ingredients, and water supplements. Figure 5 summarizes current knowledge on these three topics and how they can work to prevent disease outbreaks.

Two compounds, H$_2$O$_2$ and PAA degrade into harmless residues and are therefore more environmental friendly than other compounds. They have been proven to be effective against a variety of aquatic pathogens and represent promising approaches to treat velvet disease. Nevertheless, they could add to the stress level of the infected fish due to their high oxidative capacity and are toxic when applied at high concentrations. Conditioning the fish prior to treatment potentially helps decrease this stress.

Supplementing food with vitamins or biotics has been demonstrated to reduce stress-levels and susceptibility to infections; however, there is a risk of over-supplementation. Dose/response curve of immunostimulants does not follow a linear relationship (Bliznakov & Adler 1972). It is often a hormetic one, with beneficial effects increasing up to a certain concentration and then decreasing if that concentration is exceeded. In addition, life time, duration, and frequency of application can play a tremendous part. After continuous feeding for 40 days with glucan at a concentration of 2 g glucan kg$^{-1}$ feed, which represents optimal concentration for Asian tiger shrimp (*Penaeus monodon*), immunity was reduced (Chang et al. 2000). Indian white shrimp (*Fenneropenaeus indicus*) had highest survival rate when challenged with white spot syndrome virus (WSSV) when 2 g glucan kg$^{-1}$ was added once a week as feed supplement. Higher intervals decreased survival (Sajeevan et al. 2009). These results indicate that continuous use of immunostimulants even at an optimal dose may suppress the immunity and administration at definite intervals would give better performance resulting in enhanced survival. Clearly, more research is needed in order to identify optimal application strategies, particularly studies on fish are, to the best of our knowledge, not available.

Health benefits of natural organic matter have been proven in a plethora of different animals. Many plant extracts not only have general beneficial effects, such as enhanced growth and food conversion rate, but also increase specific and non-specific immunity. The effects of humic substances have recently been rediscovered and scientifically proven; used as feed additives, they can increase vitality.

![Figure 5](image.png)

**Figure 5** Scheme of environmental friendly treatments used to prevent or treat diseases. →: stimulation; ┴: inhibition; ?: proposed pathway.
and lifespan, stimulate growth, accelerate wound healing and increase resistance to pathogenic bacterial loads. The immune system and host-specific defense mechanisms in land vertebrates were stimulated by humic substances and they reduced susceptibility to stress and microorganisms. Conditioning fish using natural organic matter is a promising approach to enhance overall and immune-related health, making fish less susceptible to velvet disease. Ongoing research in our laboratory suggests that humic substances can increase the MALT system and thereby help in preventing diseases. Humic substances are therefore another promising approach of immunostimulation. Natural organic matter can increase host defense systems in order to decrease or even replace use of chemical therapeutants. Furthermore, humic substances are part of the natural environment of fish. In contrast to plant extracts, fish defense systems are ‘accustomed’ to these natural xenobiotics, making them ideal candidates for use in culture water.

Stimulation of immune response does not necessarily reflect an increased protection against pathogens. Studies where fish are challenged with pathogens after administration of immunostimulants are scarce and most focus on bacterial infections due to easier implementation of the experiment. Also, the higher energy demand needed to activate the immune system might even be accompanied by adverse effects such as impairment of growth. It is therefore important to monitor parameters from different regulatory pathways (metabolism, stress response, growth) in addition to the immune parameters in order to ensure safe application of new immunostimulants. Nevertheless, they have been proven to be able to help protect from stress and disease, often with the beneficial side effect of increased growth performance and have the potential to replace harmful therapeutants in aquaculture facilities. When transferring laboratory results to facilities, we highly recommend monitoring the health status of fish closely at different time points in order to ensure best output.

Practical application of immunostimulatory substances in aquaculture has just begun to be explored. The next step should be to focus on duration, frequencies and life stages as well as optimizing concentrations for different fishes in order to provide recommendations for large scale application. Pilot studies have to be conducted to evaluate effects in commercial aquaculture facilities and cope with possible problems due to upsampling from laboratory to facility devices. No strategy is without constraints; this also applies for treatment of stress and diseases in aquaculture. As demonstrated with the symbiotics, a combination of different strategies can lead to mutual assistance resulting in additive or even synergistic effects. In our opinion, the best disease management strategy would thus be a holistic one, including the physicochemical environment, the animals and the microorganisms. A favourable concept for environmentally friendly treatments against velvet disease and other fish diseases is to concentrate on two aspects: 1) treatment of the invading parasites using residue-free or naturally occurring substances, and 2) fortifying the fish to reduce susceptibility to parasites and to allow significantly reduced use of antiparasitic therapeutants. In this way, the most sustainable outcome for the environment as well as for the farmer can be achieved.

Acknowledgements

The present study was financially supported by the AiF Projekt GmbH (Federal Ministry for Economic Affairs and Energy) under project No. ZF4240502SK7. The authors thank Dr. Chuck Weirich for proofreading and his comments and suggestions to improve the manuscript. Mention of trade names or commercial products in this article is solely for providing specific information and does not imply recommendation or endorsement by the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Humboldt University, Federal Ministry for Economic Affairs and Energy, or the U.S. Department of Agriculture.

Compliances with ethical standards

The authors declare that there are no conflicts of interest. The founding sponsors had no role in the design, content or decision to publish the review.

References


Guillou L, Not F (2018) *AquaSymbio: Parasites and Endosymbioses in Aquatic Ecosystems, Biological Station of Roscoff, CNRS, France.*


Protection against pathogens - chemical treatments versus immunostimulants


LaPatra SE, Fehringer TR, Cain KD (2014) A probiotic Enterobacter sp. provides significant protection against Flavobacterium psychrophilum in rainbow trout (Oncorhynchus mykiss) after injection by two different routes. Aquaculture 433: 361–366.


Taverniti V, Guglielmetti S (2011) The immunomodulatory properties of probiotic microorganisms beyond their viability


Willford WA (1966) Toxicity of 22 therapeutic compounds to six fishes. Investigations in Fish Control.


