Hydrogent®: a new therapy in the aquarium field

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Abstract

Hydrogent® is a stabilized mix of peracetic acid and hydrogen peroxide. It may be an effective treatment against pathologies found in aquaria. Its measuring, by colorimetry or spectrophotometry, can be set up simply and effectively These techniques showed that the degradation speed of the product in water is a function of mineralisation, salinity, temperature and presence of organic matter. The disinfection potential of the two oxidizing agents, studied by ATP measuring, is immense. The therapeutical strategy may be a direct addition of the product to the treatment tank (from 1.4 to 2.8 ml/100 l) or in short baths at higher concentrations (10 minutes at 1.4 ml/10 l, 5 minutes at 2.8ml/10 l or 2.5 minutes at 5.6 ml/10 l). In all of these cases, the animals' survival rate is extremely high. The treatment destroys most of the microorganisms: bacteria, fungus, algae and viruses as well as ectoparasites of the Plathelminth type.

INTRODUCTION

With the current European legislation, traditional therapeutic tools are disappearing. Now that products like Trichlorfon or malachite green-formalin are forbidden, aquaculture and aquariology are confronting some difficult issues. In fact, the alternative solutions are rare or do not even exist. It's important to search and develop new products linked to new therapies. This context led to the experiments on hydrogen peroxide, within the "Aquarium de la Porte Dorée". Oxidizing products have been used in this establishment for many years. First tests were done with H_2O_2 (ECO-BIO 1000®) and are now conducted with Hydrogent®: a mix of hydrogen peroxide and peracetic acid.

These two products heve been known for several years as disinfectants. H_2O_2 has aiready been mentioned as an effective therapeutic tool in several references concerning aquatic pathologies. But, in the case of peracetic acid, publications dealing with its potential use in aquaculture are very rare, even if it's only used empirically.

Literature generally refers to its use in aquaculture, mainly in cold-water. Although, the aim is the same in public aquaria - the destruction of pathogen agents - conditions are different (water temperature, water circulation, decoration, vegetation or fîsh density); and the survival of some specimens is really important.

Taking account of all these specific constraints and the works already conducted, this work was divided in three parts: reactivity of the product in water, disinfection potential and therapeutic potential.

2. Materials and methods

2.1. Physicochemical characteristics of the product

Hydrogent® is a very acid (pH = 1,2) and colorless liquid, a mix of pure water (59.5%), acetic acid (25.5%) and hydrogen peroxide (15%). The two last compounds react to form peracetic acid.

Decomposition of the two compounds is characterized by the liberation of free oxygen atoms that will oxidize the organic matter and destabilize the double phospholipidic membrane of living cells. This decomposition generates acetic acid, water and dioxide, which make the product biodegradable at 90%.

2.2. Measuring techniques

Color test paper

In France, the only registered simple technique to measure out peracetic acid is Mercoquant® 1.10084, marketed by VWR International laboratories. The colorized test paper is put into the solution for one second. The acid concentration is determined by the color shown on the paper in comparison to a colored scale ranging from 0 to 50 mg/l (0/5/10/20/30/50).

But its utilization was determined to not be accurate enough in order to measure Hydrogent® concentrations.

Spectrophotometry

Quite simple and quick, this technique is marketed by VWR international, (spectroquant® 1.14731.0001) or by WTW (250402-H_20_2). It consists of taking a sample of 10 ml of the solution and pouring it into a tube containing the reactive. After three minutes, the tube is placed into a spectrophotometer that, theoretically, will read the H_20_2 concentration. The system has a precision of 0.1 mg/l from 2 to 20 mg/l.

Its main defaults are its cost and possible perturbations in reading the water concentrated with organic matter.

Colorimetry

Potassium permanganate is used to measure out the concentration of hydrogen peroxide of a solution after acidification. The solution of $KMn0_4$ is poured progressively in the sample until a persistent pink coloration is obtained. The concentration of H_2O_2 in the solution (in g/l) is obtained by the formula: 17 nt / v (with n: volume of $KMn0_4$ poured in ml; t: normality of the solution of $KMn0_4$; v: volume ofthe sample in ml).

In this experiment, the choice sample volume is 100 ml, the volume of sulphuric acid (2 N) added is 1 ml and the solution of potassium permanganate is concentrated to 0.005 N (160 mg/l).

This technique is cheaper, but time spent is longer (about 15 minutes by measuring out) and precision is less accurate.

Choice of the measuring technique

The two last techniques are theoretically marketed to measure out hydrogen peroxide. But the calculation, based on the différent measures obtained shows that the initial concentration of H_2O_2, alone, would be much higher than expected. It seems that the two techniques take the sum of the oxidant compounds in consideration. The measured value will be named as "equivalent H_2O_2" concentration, as we measure an equivalent oxidizing potential.

It's possible to determine globally the quantity of Hydrogent® in mg/l from equivalent H_2O_2: a concentration of 1 mg/l of equivalent H_2O_2 may be converted into a concentration of 0.28 ml of Hydrogent® per 100 liter of water. The concentrations obtained by colorimetry can be converted into concentrations that could be obtained by spectrophotometry. In order to do that, the following formula can be applied (Cottard 2002) :

Measure with the VWR international tube = (1.12 * measure with $KMn0_4$) - 0.42.

All results cited later will corne from a rneasuring obtained straight with a tube or from a calculation of equivalence.

2.3 Hydrogent® kinetic study

In a battery of tanks, containing 100 l of continuously stirred water, 2.8 ml of Hydrogent® is introduced in order to obtain a concentration of 10 mg/l of equivalent H_2O_2. Figure 1 shows the results obtained in osmosis water, in water of the general circulation system of the aquarium (TH = 20), in sea water and in water of the circuit added with 5 g of mashed withing. The water temperature was 25°C. An experiment with circuit water at 30°C has also been conducted.

Another experiment was set up by using filters plunged into the water circuit at 25° C, in place of the mixers. Pouzzolane, zeolite or maerl (calcified algae) grow in these filters.

2.4. ATP study about Hydrogent effects

This technique marketed by VWR international (HY-LITE 2®) is based on a reaction between ATP (Adenosin-triphosphate), from living cells, and luciferin in the presence of luciferase. The light quantity emitted during the reaction, expressed in LRU (light relative unity) and measured by a lightmeter, allows for the reading of the global contamination rate of the environment. A rapid immersion (1 sec.) of the "pen" containing the reactive products is enough to allow, by capillarity in sterile enclosure, 60 µl of liquid sample that might contain microorganisms.

3. Results and discussion

3.1. Hydrogent® kinetic

Experimental study

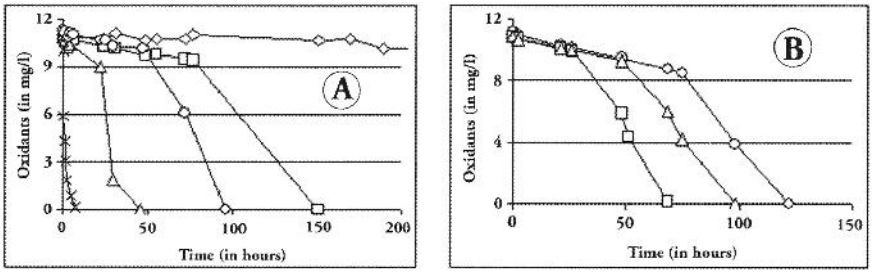

Figure 1: Evolution of the oxidants concentration (in mg/l equivalent H_2O_2). A) At 25°C, in osmosed water (rhombus), in circuit water (squares), in seawater 25 ‰ (triangles) or in water with organic matter (cross); in circuit water at 30°C (circles); B) with different kind of filtration system: pouzzolane (circles), zeolite (triangles) or maerl (squares).

Hydrogent is a stable product. At 25°C, the concentration in oxidant compounds is the same in osmosed water during at least 200 hours. But mineralisation, salinity and water temperature act on this stability. Each factor accelerates the decomposition. The filtration substrates have also an influence on the decrease of the oxidants level. This degradation takes place two times: a slow decrease and then during a quick fall. These two phases didn't appear in presence of organic matter.

Study of two cases in public exhibition tanks

This experiment is conducted on two similar tanks of 8,000 l. The first one is filled with seawater (27°C, salinity: 40 ‰) treated with 500 ml of Hydrogent® (about 30 mg/l of équivalent H_2O_2). The second one is filled with freshwater (28°C) and receives 150 ml of the same product (about 10 mg/l of equivalent H_2O_2). In these two cases, the filtration system is stopped, only the circulation water pumps still operate.

The conditions met in an exhibition tank are a synthesis of the different factors that have been studied. Hydrogent® reacts with dissolved elements, decors, substratum, plants and animals, not to mention temperature and salinity. The whole process conducts to a quick decomposition of the two

oxidants. It will then be necessary to follow precisely the kinetic decrease of the oxidants during treatment.

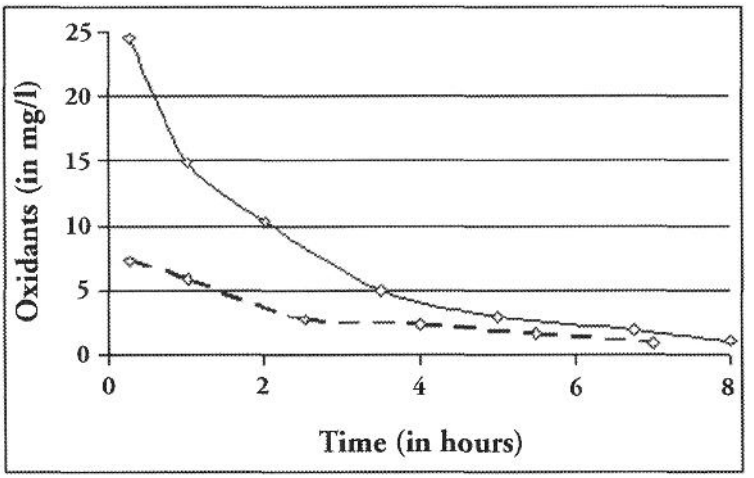

Figure 2: Evolution of the oxidants concentration (in mg/l) in two public exhibition tanks, in seawater (solid line) and in freshwater (broken line).

During the treatment with Hydrogent®, there is an increase of the redox potential. It's considered as an improvement for nocive substances elimination, like pesticides (Hwang *et al.* 2001). The same reaction was noticed in NAUSICAA (oral communication from Mr. Hénard) on a short period and in the "Aquarium de la Porte Dorée" (Hignette 1993), on a longer period, but with H_2O_2 only.

3.2. Hydrogent®, a disinfection tool

In three 100 l tanks, stirred and kept at 25°C, 4 g of withing and mashed guts is added. When the aquaria have been contaminated at similar levels, different doses of Hydrogent® are added and the evolution of the ATP is registered.

Hydrogent® reacts with organic matter. As soon as the product is added, the ATP level falls (figure 3). The higher dose of the product provokes the more important decrease. The power of the two oxidants to eliminate microorganisms has been already noticed (Stampi et al. 2001), as bactericide, virucide and fungicide.

When concentration in oxidants is weak (under 2 mg/l equivalent H_2O_2), the microorganisms left will generate an ATP level increase: the product use doesn't exempt a cleaning to destroy the inoculum left and avoid a possible recontamination.

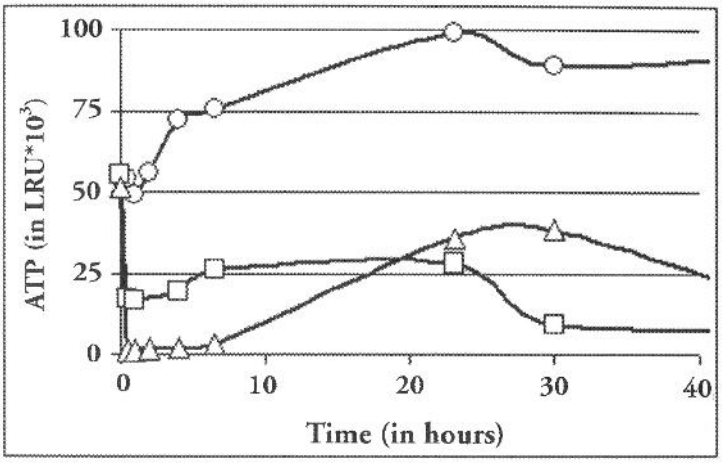

Figure 3: Evolution of the ATP level (in LRU). in a control not treated (circles), in a tank treated at 1,4 ml/100 l of Hydrogent® (squares) and in a tank treated at 2.8 ml/100 l of Hydrogent® (triangles).

3.3. Effects of the products on biological filters

Two tanks that contain 100 l of general circulation water, kept at 25°C and filtered, are added with nitrites at a level of 1 mg/l. The first one is a control, whereas the second receives a treatment dose of 2.8 ml of Hydrogent® (10 mg/l of equivalent H_2O_2). The evolution level of NO_2 - is determined during the experiment by spectrophotometry, thanks to Spectroquant® 1.14776 (VWR international).

About 230 hours are necessary for the nitrogen cycle bactena to reduce the nitrites concentration from 1 mg/l to 0. The treatment doubles this length. And, a natural increase will bring the $N0_2$ - concentration to 1.5 mg/l.

Micro-organisms activity seems reduced (Delaporte 1998), indeed totally suppressed during the first 230 hours.

Hydrogent® reacts with filtration system and reduces the number of microorganisms in the biological filters. It will then be necessary to isolate

the treated tank from its filters; unless, during the first hours, time that the major part of the oxidants disappear.

3.4. Toxicity

These tests are performed on Tanichtys albonubes. The animals are separated in shares of 30 individuals. Each share is placed in a 100 l tank with a filter. The treatment is done by direct addition of the product in the tanks with 1.4, 2.8 or 5.6 ml (14, 28 and 56 µl/l). For higher concentrations of Hydrogent®, the fish are bathed in 10 l buckets at 70, 140, 280 and 560 µl/l. Each bath is tested on different duration. Each day and during 96 hours, the dead specimens are counted and an autopsy is carried out. The aim of the manipulation is to determine the limiting Hydrogent® concentration and treatment duration.

This evaluation, started by two previous students (Chentrier 1999 and Bordat 2000), underscores a maximum in concentration and treatment durarion. For direct addition of the product in the tank, the concentrations of 14 and 28 µl/l don't endanger the fish; but with higher concentrations, some animals don't survive. For the short bath, the product (Hydrogent® concentration in µl/l) * (bath length in minutes) has to be inferior or equal to 1400 (20 min at 70 µl/l, 10 min at 140 µl/l, 5 min at 280 µl/l and 2.5 min at 560 µl/l). Exceeding this ratio, the mortality increases significantly.

The autopsies of the fish killed, by a strong treatment, show that their gills are wounded. Injuries appear at short term and look like necrosis (discoloration of the tissues). The microscopic observation reveals that rissue cohesion is broken. These results are similar to those noticed in literature about hydrogen peroxide (Tort *et al.* 1998). This paper also shows the connection between temperature and mortality.

3.5. Hydrogent®, a therapeutic tool

Facing pathogen agents:

Dactylogyrus sp.

Dactylogyrus sp. is isolated from a group of 42 piranhas {*Pygocentrus nattererî*). Nine fishes were taken and anaesthetized with phenoxy-2-ethanol. On each, a smear of the gill is performed and parasites are counted. Then, the group is separated in 7 groups of 6 individuals, placed in 100 l tanks with a filter, and treated at levels that dont implicate significant mortality. After 72 hours, each share is anaestherized and the parasites are counted.

Table 1: Average quantity of parasites per gill, before and after different Hydrogent® treatments. C: control tank not treated. ∞: long duration bath (direct addition in the tank).

Number of parasites per fish	35						
Treatment (concentration in µl/l * duration in min.)	C	14* ∞	28* ∞	70* 20	140* 10	280* 5	560* 2.5
Number of parasites per fish after 72 h	26	9	4	15	8	0	5
Survival rate after 96 h	100%						

No fish died in any therapeutical combination and all the parasites have been eliminated with a bath of 5 minutes at 280 µl/l. The Parasite quantity level falls within the treated fish while Dactylogyridae quantity remains important in the control.

Piranhas are known to be fragile fish in regards to treatments, especially to Trichlorfon. Hydrogent® represents a good alternative to more intense treatments when these kinds of pathologies occur.

Gyrodactylus sp.

The direct addition of Hydrogent® (1.4 and 2.8 ml) in tanks of 100 l containing *Poecilia reticulata*, infected by *Gyrodactylus sp.*, significantly reduces the mortality rate. But some parasites are not eliminated by these treatments at such concentrations. The product may also fight against possible additional infections.

Benedenia sp.

This experiment was performed by NAUSICAA staff, under the leadership of Mr Hénard. Two shares of 35 *Gnathanodon speciosus* infected by *Benedenia sp.* were isolated. The first one served as control and the second one was treated with 20 mg/l of equivalent H_2O_2 (56 µl/l of Hydrogent,). After 3 days, the fishes were bathed in freshwater. The flat worms that fell off during the bath were counted. Three weeks later, another bath was done and the *Benedenia* were counted again.

No parasites were collected on treated animals after the first bath, but in the following weeks, some Plathelminths were found again.

The *Benedenia sp.* create an important problem in aquariology, which concerns the major part of public aquaria, and in aquaculture, where 70% of *Seriola dumerili* may be contaminated (Wakabayashi 1996). There are one or more stages of the life cycle of the pathogen that are resistant to Hydrogent®. The solution needs to be repeated in several treatments. The

development cycle of the pathogen is about three weeks (Woo 1995); it's necessary to repeat the addition of Hydrogent, every two weeks. When, the parasite may be eliminated (Cottard 2002).

Facing non pathogenic agents: Planarians in reef tanks

In vitro, planarians are placed in Petri boxes containing a Hydrogent® solution diluted at 14, 28, l40 and 280 µl/l. The duration necessary to inactivate the totality of the flat worms is quoted. At the same time, different species of hard and soft corals are treated at the same concentrations, for a length corresponding to the inactivation of all the planarians. Then, the experiment is conducted in vivo. In a reef tank, the treatment is applied four times: at 14, then 28, 42 and 56 µl/l.

Table 2: Evaluation of planarians and corals sensibility to Hydrogent®

Hydrogent® concentration (in µl/l)	14	28	140	280
Inactivation of 100% of the planarians	after 3h	after 2h	after 10 min.	after 2.5 min.
Corals survival	yes			

At high Hydrogent® concentration (from l40 and 280 µl/l), planarians are inactivated by a short duration bath (10 and 2.5 minutes). But for lower concentrations, it's necessary to wait a few hours. In these two cases, the coralian organisms can handle the treatment well. Neither their survival, nor their growth was endangered.

The quick decrease of the product in the reef tanks containing living organisms reduces its efficiency. The experiments, in vivo, showed that doses of 14 and 28 µl/l were ineffective. And for 42 or 56 µl/l, the number of flat worms is only reduced. Conservation of Hydrogent® concentration between 14 and 28 µl/l, thanks to regular reintroduction of the product, could be an efficient therapy, harmless to the animals.

Comparison of these results with the literature is difficult: the bibliographic references generally are only concerned with the hydrogen peroxide alone. It allows, at high concentration, to treat ectoparasites (Mischke et al. 2001, Montgomery-Brock et al. 2001), bacteria (Lumdsen et al. 1998) and fungus (Fitzpatrick et al. 1995). But environmental conditions are not similar in aquariology. However, some papers do exist about Detarox®, a mix of hydrogen peroxide and peracetic acid, close to Hydrogent®. This

product is evocated as a fungicide and bactericide (Rahkonen and Koski 2002). It is presented too as an efficient tool against the parasite *Trichodina sp*. (Madsen et al. 2000) and as a virucide (Technical report from the Danish laboratory for fish diseases 1999).

5. Conclusion

Hydrogent® appears as an efficient alternative to the forbidden therapeutical products. Its effîciency is similar to the other products used today and it is able to destroy numerous pathogens.

Its biocide effect allows for thought about its use to purify aquarium water. Its properties provide many possibilities to destroy the noxious microorganisms. Bactericide, algaecide, fungicide and antiparasite: its characterisrics allow for the fish to survive most ofthe biological attacks in aquarium.

Its use can be preventive. Product addition in quarantine tanks stops the development of undesirable hosts. It can be added directly to exhibition tanks. In this case, it will be necessary to isolate the fîltration system, for a few hours, to preserve the biological filters. Moreover, it doesn't cause any visual annoyance for the visitors, thanks to its absence of coloration. Finally, the short bath at a high concentration (recommended at 280 µl/l during 5 minutes) is a tool for parasite destruction and for external lesions disinfection. A controlled use of the product allows for the maintenance of fîsh and fragile species, as corals.

Its quick decomposition leaves a clean environment, limiting the long-term side effects. Hydrogent® is a product with a future, at least in aquariology and perhaps in aquaculture.

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