

Negative effects of malachite green and possibilities of its replacement in the treatment of fish eggs and fish: a review

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ABSTRACT: Malachite green has been used as an effective compound to control external fungal and protozoan infections of fish since 1933 but it has never been registered as a veterinary drug for use in food fish because of its potential carcinogenicity, mutagenicity and teratogenicity in mammals. The present paper reviews negative side-effects of malachite green including its accumulation and persistence in fish that have been treated and describes other alternative substances for the treatment of fish and fish eggs.

Keywords: malachite green; fish baths; toxicity; accumulation; residues in fish; aquatic environment; protozoan ectoparasites; fungal infections

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1. Introduction

Until recently, malachite green belonged to the most frequently used disinfectants, and, especially in salmonids farming, it was considered as practi-

cally irreplaceable. Fungicidal effects of malachite green have been known since the mid-1930s (Foster and Woodbury, 1936). In the 1950s, malachite green was used as a very effective antiseptic, and against both internal and external parasites. In the 1960s,

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malachite green proved to provide the most effective treatment against protozoan ectoparasites, particularly *Ichthyophthirius multifiliis*. Malachite green became even more important when its effectiveness against water fungi *Saprolegnia* sp. in fish eggs (Olah and Farkas, 1978; Alderman and Polglase, 1984) and its applicability for the treatment of proliferative kidney disease of salmonid fish (Clifton-Hadley and Alderman, 1987) were demonstrated. In the Czech Republic, malachite green was most frequently used to treat cases involving *Ichthyobodo necator*, *Trichodina* sp., *Trichodinella* sp., *Chilodonella* sp. and *Ichthyophthirius multifiliis*, and skin fungal infections of fish and fish eggs. A short-term malachite green bath was also recommended as a treatment of gill flavobacteriosis in salmonids (Citek et al., 1997).

Malachite green is a basic dye, readily soluble in water. In fish breeding, technical grade malachite green was used in the past. Its exact chemical composition was not often given. When using it, it was therefore necessary to take into account its different toxicological and therapeutic properties. The malachite green bath treatment without a prior test of fish tolerance could result in the death of all the stock thus treated. For that reason, each new production batch of malachite green had to be tested for toxicity to fish and for its antiparasitic action.

Malachite green was used for monocomponent treatment baths (only malachite green at various concentrations) as well as for multicomponent treatment baths (malachite green in combination with formaldehyde, brilliant green, crystal violet, methylene blue, etc.).

Scientific evidence indicated that malachite green (MG) and especially its reduced form, leucomalachite green (LMG), might persist in edible fish tissues for extended periods of time (Mitrowska and Posyniak, 2004).

In 2000, the use of malachite green for food fish was banned in the EU because the general public may become exposed to malachite green through the consumption of treated fish. Concern about the potential for human toxicity and the presence of malachite and leucomalachite green (a metabolite) in the aquatic environment has resulted in increased monitoring especially in food supplies.

Other toxicological tests with malachite green were carried out on warm-blooded animals where carcinogenicity and teratogenicity were demonstrated. A threat to human health was confirmed by that time only in direct contact and manipulation

with malachite green, when it causes the irritation of eyes (Grant, 1974). At present, this agent can be used only in aquarium and ornamental fish breeding.

2. Preventive and curative malachite green baths

2.1. Curative fish baths

In aquarium and ornamental fish rearing, dips, short-term baths and long-term baths are used.

Dips are of 10 to 30 s in duration, and the recommended concentration is within the range of 66.7–100 mg/l. Dips are used to treat topical fungal infections in fish (Nelson, 1974; Citek et al., 1997).

Short-term malachite green baths are treatments 1 to 1.5 h in duration at the bath concentration of 6.7 mg/l. If water temperature is below 10°C, baths may take place in different types of tanks. If water temperature exceeds that limit, baths should be conducted only in holding tanks that can be easily filled with water and emptied within 30 min (Citek et al., 1997).

Long-term (six-day) malachite green baths used to be performed in holding tanks to which enough malachite green was added to obtain a final concentration of 0.5 mg/l in the cyprinids (Svobodova et al., 1997). Somewhat lower concentrations (0.15 to 0.20 mg/l) were used in the salmonids. Following the application of malachite green and its uniform distribution in the tank, the flow of water through the tank was stopped, and adequate aeration had to be provided for. After 24 h, the bath water was drained, clean water was allowed to flow through the tank for an hour, the tank was then refilled with water and another dose of the dye was applied. This procedure was repeated six times in total (Citek et al., 1997). This type of bath was used to control protozoan ectoparasites, particularly the ciliated protozoan *Ichthyophthirius multifiliis*.

In an effort to reduce concentrations of, and exposure periods to, malachite green, monocomponent malachite green baths were gradually replaced with multicomponent baths. A combined malachite green and formaldehyde bath [0.25 mg malachite green and 0.125 ml 36–38% aqueous solution of formaldehyde (hereinafter only “formalin”) dissolved in 1 l of water] appeared very effective. The treatment period depended on available technological facilities and might be 2 h in aerated laminated troughs or 6 hours in holding tanks. When treating

fish with ichthyophthiriosis in laminated troughs, it was recommended to repeat baths 2–3 times a week (Citek et al., 1997).

The most favourite preparation of aquarists is a mixture of formalin, malachite green and methylene blue known as FMC (3.5 g malachite green and 3.5 g methylene blue in 1 000 ml formalin). Curative baths are prepared by adding 1.5–3 ml FMC to 100 l water (Bassleer, 1983). The overall procedure takes three days, and every day a new curative bath must be prepared. The procedure is mainly used to control parasites of the genera *Cryptobia*, *Ichthyobodo*, *Chilodonella*, *Trichodina*, *Trichodinella*, and skin fungal infections.

In his experiments, Lanzing (1965) demonstrated bacteriostatic effects of a bath consisting of three components, i.e. 0.8 g malachite green, 0.8 g brilliant green and 0.6 g crystal violet in 10 l of water. The dips lasted 10 s and were repeated two or three times a week.

2.2. Curative baths for fish eggs

For preventive baths of ornamental and aquarium fish eggs, malachite green was mainly used in the FMC formulation.

For the treatment of carp eggs infected with fungi *Saprolegnia* sp., 1-hour treatment in malachite bath at the concentration of 4–5 mg/l proved effective. In the Czech Republic, fish egg treatments at concentrations of 5–10 mg/l were mainly recommended (exceptionally 50 mg/l) applied for 5–30 min, once or twice daily, or twice weekly. The use of malachite green was prohibited for the treatment of eggs of herbivorous fish and tench (Citek et al., 1997) because eggs of those fish species are very sensitive to malachite green. Bath treatment for salmonid fish eggs was described by Willoughby and Roberts (1992).

3. Safety risks posed by the use of malachite green in fish rearing

In spite of the excellent curative properties of malachite green, we cannot leave out its significant negative properties, including:

3.1. High acute toxicity to fish

Malachite green is highly toxic to fish. Lethal concentrations for fish and recommended thera-

peutic concentrations are sometimes very close to each other (Svobodova and Vykusova, 1997). The progress of intoxication is very rapid and very much alike in both the common carp and rainbow trout. Typical clinical symptoms include restlessness and uncoordinated movements of the fish in the tank. The fish move in the upper half of the tank, leap above the water surface, and carp gasp for air, which is followed by the loss of balance, apathy, agony and death. The pathological anatomical picture of fish intoxication with malachite green is characterized by greenish tinge of their skin and increased production of skin slime. The gills are oedematous, with excessive amounts of mucous matter, and are discoloured by the agent. Vessels in the body cavity were dilated, and muscle tissues and internal organs were often light-green in colour (Machova et al., 2001).

Large differences in the toxicity of malachite green in dependence on its purity and varying concentrations of residual impurities that could render more or less toxic are another major obstacle in its application. High-quality grades of malachite green can be produced by the inclusion of additional purification stages in production, but even a nominal 100% malachite green dry powder by analysis can only contain 82% (oxalate) or 95% (hydrochloride), the rest of the weight being the acid component (Alderman, 1985). Different toxicological properties of malachite green were confirmed in a series of acute toxicity tests on common carp and rainbow trout in which 10 types of malachite green obtained from different sources were investigated. It follows from these experiments that lethal concentrations of some types of malachite green are very close to therapeutic concentrations. In one case the recommended therapeutic concentration of malachite green was even higher than its lethal concentration (Svobodova and Vykusova, 1997).

When preparing bath solutions, it should be borne in mind that malachite green toxicity is significantly influenced by the quality of used water. The bath toxicity and hence of course its effectiveness are primarily influenced by the reducing substances present in water, for example organic substances, ions and pH values (Alderman, 1985; Mitrowska and Posyniak, 2005). The toxicity of malachite green is reduced for example by humic substances (HS) in soft water. Because HS alter the toxicity of malachite green, the therapeutic index might also be influenced by HS. Whereas in the presence of high concentration of Ca, the binding

sites of HS are filled with Ca-ions and these cause the lower binding of malachite green to HS. Therefore, the toxicity as well as the efficacy of the malachite green treatment may be altered in the presence of HS and Ca content in water (Meinelt et al., 2003). Tolerance testing prior to bath treatment was always necessary. Water used in the test had to be the same as water used for bath treatment itself, and the same stocking densities of fish had to be used, if possible.

For the above reasons, it was important to pay great attention to the selection of malachite green to be used, take water parameters and temperature into account and also observe recommended dosages and exposure periods, which could be different for distinct species as well as for different developmental stages of fish (Theron et al., 1991).

3.2. Side-effects on treated fish and fish eggs

In addition to its high acute toxicity, malachite green may be the cause of a number of side-effects on the fish treated.

Trout treated with a 1-hour malachite green bath at the concentration of 2 mg/l showed lower weight gains, higher mortality and overall anaemia later in their life. The brown trout treated with malachite green in their early stages showed a higher incidence of tumours in the abdomen, intestines and liver (Mayer and Jorgenson, 1983).

Tests on cyprinid fish demonstrated that malachite green at a concentration of 0.1 mg/l produced a cytostatic syndrome affecting the chromosome division in experimental fish (Keyl and Werth, 1959). In tissue culture tests involving a 96-hour exposure to malachite green at concentrations from 0.5 to 20 mg/l cytopathic changes, partial cytoplasm vacuolization, granulation and disintegration of a majority of cells were recorded (Studnicka et al., 1977). The application of malachite green had a negative effect on the regeneration of damaged gill epithelium. Histopathological examinations of fish following a malachite green bath treatment showed generalized activation of the reticuloendothelial system (RES) and incidence of inflammatory cells in spleen. Increased haemosiderosis in spleen and kidneys was observed 14 days after a malachite green treatment of fish (Rehulka, 1977).

In carp treated with malachite green, histopathological examinations revealed moderate regressive changes on gills, moderate dystrophic changes in parenchymatous tissues and increased macrophage

activation (Svobodova et al., 1997). Hyperaemia and focal necrotization of liver were observed in rainbow trout after three 40-min malachite green bath treatments at a concentration of 6 mg/l. Mitochondria damage and endoplasmic reticulum dilation were found at the ultrastructural level. Other findings on the gills of treated fish included separation of epithelial cells, leukocytary infiltration, and an onset of lamellar cell necrosis. The frequency of these changes increased with the number of bath treatments (Gerundo et al., 1991; Srivastava et al., 1998).

A malachite green bath treatment also affects the blood count of treated fish. In common carp, a six-day curative malachite green bath treatment at a concentration of 0.5 mg/l caused significant changes in both red blood cell count (decreased haematocrit value, decreased erythrocyte volume, increased mean corpuscular haemoglobin concentration) and white blood cell count, which showed a decrease in both the absolute and the relative numbers of monocytes. A decrease in the blood plasma total protein concentration was also ascertained (Svobodova et al., 1997). Similar results were also reported by other authors (Srivastava et al., 1995; Tanck et al., 1995; Saglam et al., 2003). A long-term exposure of African walking catfish (*Clarias gariepinus*) to malachite green caused anaemia and a reduction in the number of neutrophil granulocytes (Musa and Omoregie, 1999).

A malachite green bath treatment of rainbow trout eggs delayed the time of sack fry hatching by 5 to 8 days compared with controls. The larvae hatched from treated eggs exhibited an increased frequency of abnormalities (malformation of the head and jaws, spine deformation or missing fins). Although the percentage of eggs that reached the eyed eggs stage after malachite green treatment increased compared with the controls (probably due to reduced fungal infestation of the eggs as a result of the treatment), the increase did not compensate for the loss of larvae due to malformations caused by malachite green treatment (Mayer and Jorgenson, 1983). Fish eggs treated with malachite green also exhibited mitotic defects and chromosomal break points (Mayer and Jorgenson, 1983).

3.3. Accumulation and persistence in treated fish

Malachite green exhibits high affinity to animal tissues. During bath treatment, Bauer et al. (1988) observed a marked decrease in the malachite green

concentration in the bath and its accumulation in the fish. According to literary data, up to 90% of malachite green absorbed by the muscle tissue is accumulated as leucobase (reduced colourless form) and as this form it persists in fish for a very long time (Bauer et al., 1988; Plakas et al., 1996; Alborali et al., 1997). An extremely high concentration of malachite green in fish tissues was detected after a combined bath (1 mg/l malachite green and 0.02 ml/l formalin) (Clifton-Hadley and Alderman, 1987).

The main malachite green excretion pathways in rainbow trout are the liver and the gall bladder. The highest concentrations of leucobase-persisted residues were found in liver (Alderman and Clifton-Hadley, 1993).

The time from bath treatment until the residues drop to an undetectable concentration depends not only on the initial malachite green concentrations in the fish but also on their growth rate. In market-ready fish that will not grow any bigger, a longer time (up to three times) was needed for the elimination of leucobase residues than in fish with a short period of growth (12 months) (Bauer et al., 1988). Data on malachite green persistence in the muscle tissue of various species of fish were also published by Mitrowska and Posyniak (2005) (Table 1).

The Czech Republic had no definite allowable level for a malachite green concentration in food fish, but bath treatment instructions, valid until the year 2000, recommended that a six-month protective period between the treatment and the marketing of fish had to be observed.

In the Czech Republic, 6-day malachite green baths at 0.2 mg/l concentration were very frequently used in rainbow trout rearing facilities. An experiment including a six-day bath of rainbow trout was therefore conducted to find whether the recommended six-month withholding period between the bath and the marketing of the fish was sufficient. Results of the test confirmed that malachite green was highly cumulative. It was also demonstrated that immediately after a long-term bath malachite green concentrations in the muscle tissue of the treated fish exceeded the 700 µg/kg concentration and that the recommended six-month withdrawal period between the bath and the retail marketing of fish was absolutely insufficient (in the muscle of these fish, 21 to 88 µg/kg of malachite green sums was detected six months after the bath). All samples of tested muscle tissue, liver and skin were negative (< 5 µg/kg, i.e. below the detection threshold of the previously used analytical method) only 12 months after the bath (Machova et al., 1996).

Table 1. Persistence of malachite green (the coloured form MG and the leuco form LMG) in the muscle tissue of various fish species after bath (Mitrowska and Posyniak, 2005)

Fish species (average body weight)	Concentration of MG (mg/l)	Duration of bath (hours)	Water temperature (°C)	pH of bath	Time after bath (days)	Concentration in muscle tissue (µg/kg)	
						MG	LMG
Eel (4.1 g)	0.10	24	25	6.9	62	2	139
					80	bdt	28
					100	bdt	15
Eel (0.3 g)	0.15	24	25	6.9	330	bdt	bdt
Channel catfish (600 g)	0.80	1	21	7.1	14	12	518
					42	bdt	19
Channel catfish (580 g)	0.80	1	22	7.2	14	6	310
Rainbow trout (1 350 g)	1.00	1	21	7.0	1	73	289
Rainbow trout (1 350 g)	1.00	1	12	7.8	5	15	230
Rainbow trout (0.1 g)	0.20	72	9,7	–	40	1	20
					140	bdt	
Rainbow trout (0.1 g)	0.20	144	13	–	300	bdt	2

dt = below the detection threshold of the analytical method

3.4. Residue findings in harvest-size fish

Resolution (EEC) No. 2377/90 of the European Council imposed a strict ban on the use of malachite green in all age categories of food-producing fish (i.e. including fish eggs). This ban came into effect on 1 January 2000. In 2002, the Commission of European Communities approved Decision No. 2002/657/EC, in which it laid down the so-called minimum required performance limit (MRPL) for analytical methods to be used for determining substances that are either not approved for use in the EU states or are explicitly banned. The MRPL for malachite green aggregate concentration was set at 2 µg/kg.

Because the use of malachite green in food-producing animals was banned but there was no adequately efficient preparation available (and there still is none), it was only to be expected that the malachite green ban will not be strictly observed by fish farmers. This assumption has been confirmed. In the Czech Republic, the first problems in connection with positive results of tests for malachite green in fish exported to Switzerland arose in 2000. In 2001, checks of harvest-size rainbow trout randomly purchased from seven different commercial trout farms in the Czech Republic were made. The fish from all the farms were tested positive in the sum of malachite green (MG and LMG) analysis. While malachite green sums in fish from three farms were from 5 to 10 µg/kg, they ranged between 40 and 102 µg/kg in fish from the other four farms. This fact prompted the State Veterinary Service of the Czech Republic to make further inspections targeted specifically at the determination of malachite green residues. Results of the tests are published annually in Information Bulletins "Contamination of Food Chains with Exogenous Substances" (<http://www.svscr.cz>). According to these sources, a total of 156 fish samples were tested in the Czech Republic in 2002 (66 carp samples, 62 rainbow trout samples, six brown trout samples, six tench samples, and other fish species accounted for 10%), of which 17 rainbow trout and one common carp were tested positive (6–208 µg/kg and 11 µg/kg of the sum of malachite green, respectively). The tests of 37 freshwater fishes carried out in 2003 returned four positive results. Positive tests were for one silver carp (9 µg/kg), one imported brown trout (13 µg/kg) and two rainbow trout (11 and 73 µg/kg, respectively). In 2004, 20 carp samples were examined with negative results and all 13 rainbow trout examined were found positive (2–6 µg/kg).

The situation in fish contamination with malachite green in other countries was described by Mitrowska and Posyniak (2005). In their paper, the authors published results of their investigations into malachite green residues in fish in the EU member states. It follows from their results that malachite green concentrations in excess of the permitted limit (2 µg/kg) were recorded in 112 fish samples in 2002, and that the largest numbers of positive tests were in Ireland (32) followed by France (27), Austria (18) and the UK (16). In 2003, the number of positive results dropped to 81, and most of them were from the UK (29), followed by France (14), Ireland (9) and Austria (9). In Poland, 10 checks were made in 2003 with one sample tested as positive, and in 57 checks made in 2004 thirteen samples of trout, carp and grass carp muscle were tested as positive. Because, however, no specific analytical results are given in the reports, it is not possible to determine how much the limit value was exceeded.

3.5. Effects and persistence of malachite green in the aquatic environment

The literature gives relatively few data on negative effects of malachite green in the aquatic environment. Malachite green persistence of about 80 days is usually given for the natural aquatic environment. It is degraded *inter alia* by hydrolysis (Anonymous, 1999). The effects of malachite green on aquatic organisms (aquatic invertebrates and algae) have not been described and documented sufficiently yet.

Malachite green can be removed from water by a number of chemical, physical and biological technologies.

Chemical methods including reductive and oxidative degradation are effective only when the solute concentration is relatively high. The required electrochemical treatment and high dosage of chemicals may also make the process uneconomical (Kumar et al., 2006).

The most widely used method is activated carbon and coal adsorption (Marking et al., 1990; Aitchison et al., 2000; Singh et al., 2003). The filtration of contaminated water through granulated active charcoal filters can reduce the malachite green concentration from 1 ppm to levels below 0.1 ppm. The filtration efficiency depends on the size of active charcoal granules and on the water flow rate (Marking et al., 1990; Marking, 1991).

This method does not degrade the pollutants, but only transfers them from the liquid phase to the solid phase, thus causing secondary pollution or requiring regeneration that is a costly and time-consuming process (Tan et al., 2006). Crini et al. (2007) summarized a number of other non-conventional sorbents, which were studied in the literature for their capacity to remove malachite green from aqueous solutions, such as sugarcane dust, sawdust, bottom ash, fly ash, de-oiled soya, maize cob, peat, iron humate, mixed sorbents, activated slag, waste product from agriculture, bentonite, and magnetic nano-particles.

Malachite green may also be removed by biosorbents. In the last years, a number of studies have focused on some microorganisms that are able to decolorize, biosorb or biodegrade dyes in wastewaters. The ability of biosorption and also partial biodegradation was described on microorganisms, including microalgae *Cosmarium* sp. (Daneshvar et al., 2007a), *Chlorella*, *Chlamydomonas*, *Euglena* (Daneshvar et al., 2007b), bacteria *Kurthia* sp. (Sani and Banerjee, 1999), *Citrobacter* sp. (An et al., 2002) and fungi *Formes sclerodermeus*, *Phanerochaete chrysosporium* (Papinutti et al., 2006) and *Pithophora* sp. (Kumar et al., 2006). The use of biomaterials as sorbents for the treatment of wastewaters will provide as a potential alternative to the conventional treatment (Kumar et al., 2006).

3.6. Toxic effects of malachite green in mammals

The basic chemical structure of malachite green and its metabolites (Figure 1) signals a degree of carcinogenicity. Malachite green is transformed in organisms to leucomalachite green, which accumulates in the tissues of exposed organisms from where it can easily get into the human food chain. Both clinical and experimental observations reported so far reveal that malachite green is a multi-organ toxin (Srivastava et al., 2004). Malachite green belongs to the same group of triphenylmethane dyes as crystal violet, in which carcinogenic effects have been demonstrated. Based on the same group classification, a carcinogenic effect can be assumed (Au et al., 1978; Culp and Beland, 1996).

The median lethal dose (LD_{50}) for malachite green in rats is 275 mg/kg after peroral application, and more than 2 000 mg/kg in the case of dermal exposures. The acute toxicity of LMG is unknown, but it is assumed to be about ten times lower than that of the initial ion or carbinol forms of MG (Clemmensen et al., 1984).

Toxic effects of the dye (MG and LMG) have been tested on mice and rats in 28-day administration experiments. It was demonstrated that the tested substances showed affinity to liver, thyroid gland and bladder, where morphological changes were observed. The highest level of malachite green at

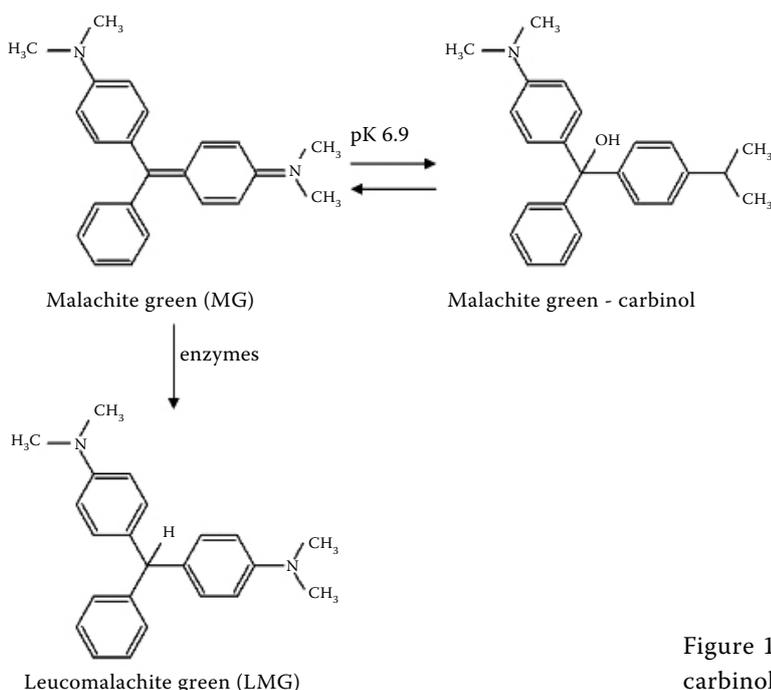


Figure 1. Structural formula of malachite green, its carbinol form and leucomalachite green metabolite

which no statistically significant adverse effects on the organism were observed compared with the control group (NOAEL) was found to be 8 mg/kg in rats and 40 mg/kg in mice. The NOAEL of the leucomalachite green was found at 23 mg/kg for rats and 77 mg/kg for mice. It follows from the results that mice are less sensitive to the effects of the dye. Contrary to the original assumption based on the structural arrangement, relatively higher toxicity of leucomalachite green and its ability to form DNA adducts in the liver, and thus also the potential for carcinogenic effects, were demonstrated (Srivastava et al., 2004).

Laboratory tests also demonstrated that malachite green may damage DNA after *in vitro* metabolic activation, although no genotoxicity was demonstrated in *in vivo* tests. No genotoxic effects induced by leucomalachite green were demonstrated *in vitro*, and malachite green effects *in vivo* were confirmed only by the positive results of tests on transgenic mice. These findings suggest a low or even doubtful genotoxic potential of the two compounds (Fessard et al., 1999).

Malachite and leucomalachite green carcinogenicity was studied in two-year tests on rats and mice. Carcinogenicity changes were observed in the liver of mice fed leucomalachite green at 31 mg/kg (Culp et al., 2006).

Thus there arises a question why the leucoform of malachite green produces nodules in the liver of only mice while most of the tests assessing the formation of DNA adducts showed that the rats were the more sensitive species, where, however, the administration of leucomalachite green failed to induce any carcinogenic division. On the other hand, Werth (1958) reported a marked increase in the number of tumours in internal organs of rats that were fed a diet containing malachite green. Werth (1958) also reported defects in up to nine generations of the progeny of rats after oral application of 1 mg malachite green, which is also contradictory to conclusions drawn by Fessard et al. (1999). Although it is difficult to pronounce on any dependence between genotoxic and carcinogenic properties on the basis of the information presented, malachite green nevertheless appears to be a promoting agent in the formation of nodular lesions (Culp et al., 2006).

Based on results in rabbits, malachite green is classified among substances with teratogenic effects (Meyer and Jorgenson, 1983). It has also been demonstrated that MG is able to inhibit mi-

tochondrial oxidasis and glutathion-S-transferase (Glanville and Clark, 1997), and that LMG is able to inhibit thyroidal peroxidase, whose activity determines the thyroid hormone synthesis (Doerge et al., 1998). Besides exhibiting a highly cytotoxic action on all mammalian cells, malachite green also induces lipid peroxidation and the formation of dangerous free radicals (Srivastava et al., 2004).

Information on genotoxicity, bioaccumulation and reproductive genotoxicity is still incomplete, and neither have the admissible daily intake (ADI) limits been set.

4. Alternative use of other substances for the treatment of fish eggs and fish

Malachite green has gradually been replaced in the treatment of superficial fungal infections and infections with protozoan parasites with the exception of *Ichthyophthirius multifiliis*.

In treatments of topical mycoses, malachite green is being replaced mainly by formaldehyde, potassium permanganate and bronopol (2-bromo 2-nitropropane 1,3 diol) baths (Citek et al., 1997; Cawley, 1999; Pottinger and Day, 1999). Sodium chloride and chloramine have been successfully used in the treatment of protozoan infections and flavobacteriosis of the gills in salmonids, respectively.

4.1. Treatment of fish eggs

In some EU states, Pyceze solution was registered for treatment baths for food fish eggs (<http://www.vmd.gov.uk/espcsite/Documents/131969.DOC>).

The preparation was developed in Great Britain in cooperation between salmonid fish producers and the Department for Environment, Food and Rural Affairs (DEFRA), and it is manufactured by Novartis Animal Vaccines. Its active ingredient is bronopol (2-bromo 2-nitropropane 1,3 diol), a member of the aliphatic halogenitro compounds, routinely used as an antimicrobial preservative. It is active against the cell wall and membranes of metabolically active bacteria and also fungi. Pyceze is particularly recommended for the treatment of salmonid fish eggs, and for the treatment and control of fungal infections (*Saprolegnia* sp.) in rainbow trout and Atlantic salmon. Pyceze is not recommended for the treatment of rainbow trout alevins or smolting Atlantic salmon because increased toxicity of

the preparation to these developmental stages was demonstrated. Pyceze is degraded by prolonged or repeated exposure to UV light, which may alter its toxicity profile. It is therefore recommended not to pass Pyceze treated water repeatedly through ultra-violet sterilizing filters.

Eggs are treated once daily at 50 mg/l Bronopol for 30 min, commencing 24 h after fertilisation at the earliest. Fish are treated at 20 mg/l Bronopol for 30 min once daily, for up to 14 consecutive days. Pyceze (Bronopol) may also be used to advantage against bacterial diseases, such as the rainbow trout fry syndrome (RTFS), i.e. anaemic syndrome of the rainbow trout fry caused by *Flavobacterium psychrophilum*, bacterial gill disease (*Flavobacterium* sp.) and other bacterial diseases. We should, however, bear in mind that the preparation is irritating to eyes, lungs and skin, and the use of protective equipment is necessary. Pyceze can be imported to the Czech Republic and applied here based on the approval by the State Veterinary Service.

Baths of 36% to 38% formaldehyde (formalin) solutions at concentrations from 0.05 to 0.35 ml/l for 10 min once in every two hours are used to treat eggs of herbivorous fish. Formaldehyde baths can also be used for the treatment of eggs of other fish species (Citek et al., 1997).

In the treatment of eggs of tench and other cyprinid fish, malachite green may be replaced with iodine-detergent preparations Wescodyne and Jodisol (with poly-N-vinylpyrrolidon-2 as the active agent). These preparations are effective against fungi, bacteria and viruses that may be transmitted by fish eggs. Wescodyne is used at concentrations of 2 to 20 ml/l, Jodisol at 2 to 50 ml/l, exposure times of both the preparations range from 2 to 5 min once or twice daily in a broad range of fish species (Citek et al., 1997).

To treat eggs of salmonid and coregonid fishes, dipping in solutions of 20 to 50 g/l sodium chloride or 0.5 g/l acriflavine for 20 to 30 min can also be used. Acriflavine is also used for the treatment of aquarium fish eggs (Citek et al., 1997).

The need of chemotherapeutics can be minimized by the use of safe groundwater (free of fungal germs, etc.) for egg incubation. It means that, first and foremost, good quality water needs to be provided. If no source of such quality water is available, it is recommended to disinfect the supplied water by UV light (e.g. by installing a UV lamp in the supply pipe). UV light effectiveness may however be markedly reduced in water con-

taminated by undissolved particles. In aquarium rearing facilities, water disinfection by ozone has been used to advantage (the presence of ozone in atmosphere requires that adequate labour safety and health protection measures for the staff need to be in place) (Citek et al., 1997).

4.2. Fish treatment – treatment of ichthyophthiriosis and fungal infections

One of the alternative, albeit technically prohibitive, methods of treating ichthyophthiriosis is a temporary increase in water temperature. The treatment consists in increasing the temperature of water with infected fish to 31–32°C and maintaining it there for three days. After three days, the water is allowed to gradually cool to the original temperature. The fish population gets rid of the infection, and acquires a considerable degree of immunity in the process. This method is used in aquarium fish rearing and in special fish-rearing facilities with heated water (Citek et al., 1997).

Ichthyophthiriosis can also be successfully treated either by placing the fish in another tank or by keeping the fish in a strong stream of water for some time. Moving the fish to another tank has proved successful for the treatment of ichthyophthiriosis in gold fish of reasonable good health. The fish were moved daily for three days. On day four, only very few *Ichthyophthirius multifiliis* were found. Ichthyophthiriosis was also largely controlled in rainbow trout kept in a strong stream of water for 10 days. The fish were fed spleen to keep them in good condition (Svobodova et al., 1995).

Following the ban on the use of malachite green in the EU, a seminar on malachite green substitutes and alternative strategies against fungal infections and ichthyophthiriosis was organised at the 10th International Conference of the European Association of Fish Pathologists (EAFP) in Dublin (Rahkonen et al., 2002). The results of a questionnaire sent to the Finnish producers of salmonid fish were presented there where they were asked to compare the effectiveness of Leteux-Meyer mixture (malachite green and formalin) with that of other preparations. Short-term formalin baths (60 or 250 mg/l for 60 or 20 min), sodium carbonate peroxyhydrate baths (60–90 mg/l, 30–60 min, daily for 4–6 days) or baths in a mixture of acetic acid, peracetic acid and hydrogen peroxide (Detarox, 10 mg/l 25–40 min, second treatment

after 4–6 days) were evaluated for the most part as less effective than malachite green treatment. The same or better results were achieved with copper sulphate dip baths (250–500 mg/l for 30 s). The problem of copper sulphate is its high toxicity and the fact that dip baths are very labour intensive to organize.

Another alternative is the use of metronidazole (humane preparation Entizol) or dimetridazole in the feed. Metronidazole is applied at a concentration of 7.5 mg/kg fish per day for a week. A dimetridazole dose is 56 mg/kg fish daily for 10 days (Citek et al., 1997). Neither metronidazole nor dimetridazole are, however, registered for use in food animals in the EU. For that reason, they can only be used to treat non-food fish (Rahkonen et al., 2002). These approaches proved successful only in fish that readily accepted medicated feed.

Scottish authors compared the effectiveness of six ichthyophthiriosis suppressing chemotherapeutics included in the feed. Amprolium hydrochloride ($C_{14}H_{19}N_4^+Cl^-HCl$) is in competition with the active transport of thiamine. Clopidol ($C_7H_7C_{12}NO$) blocks the release of coccidial oocysts. Decoquinat ($C_{25}H_{35}NO_5$) interferes with cellular respiration. Monensin ($C_{36}H_{61}O_{11}Na$) affects mitochondrial activity. Nicarbazin ($C_{19}H_{18}N_6O_6$) inhibits energy metabolism. Salomycin sodium ($C_{42}H_{69}NaO_{11}$) activity is similar to that of monensin. Tests with rainbow trout fry have shown that the use of amprolium hydrochloride (104 mg/l) and clopidol (65 mg/l) 10 days prior to experimental infection reduced the number of trophozoites by 62% and 35.2%, respectively. In curative treatment, chemotherapeutics mixed into feed were administered for 10 days. Amprolium hydrochloride (63 mg/l and 75 mg/l) reduced the number of surviving trophozoites by 77.6% and 32.2%, respectively, clopidol (92 mg/l) reduced the number by 20.1% and salomycin sodium (38, 43 and 47 mg/l) reduced the numbers of trophozoites by 80.2%, 71.9% and 93.3%, respectively (Shinn et al., 2003).

The ichthyophthiriosis treatment in trout was also tested in field trials on two Finnish farms in fish kept in concrete tanks. The preparations tested in the experiments included formalin, potassium permanganate ($KMnO_4$), chloramine, hydrogen peroxide (H_2O_2) and preparations Per Aqua and Desirox, which are a combination of acetic acid and hydrogen peroxide. All of these preparations were tested individually or in combination with formalin. It was demonstrated that the use of those

preparations or their combinations may successfully reduce the fish infestation with parasites to such an extent that high mortality is prevented in the first four weeks of infection, the immune response is meanwhile induced and the treatment can be discontinued. The combination Desirox/formalin proved to be the most effective (Rintamaki-Kinnunen et al., 2005a).

Experiments similar to those conducted by the Finnish authors were carried out with salmonids in in-ground ponds where the situation is more complicated compared with concrete tanks. In three experiments on Finnish farms, formalin and formalin/chloramine and formalin/Desirox combinations were used. When all three preparations were used, parasites were eliminated after four to five weeks of treatment. The Desirox/formalin combination was not however more effective than the others. This was probably so because the organic contamination in in-ground ponds is greater than in concrete tanks, which may reduce the effectiveness of the preparation (Rintamaki-Kinnunen et al., 2005b).

Besides the above-mentioned Pyceze, preparations used for the control of ichthyophthiriosis in Austria include Perotan Aktiv (FIAP aquaculture), which is described by the producer as an alternative to chloramine (the composition of the preparation is not given). Perotan Aktiv is used at a concentration of 10 mg/l and it is applied on days 1, 3, 5, 7, 12 and 14.

When searching for malachite green substitutes, *in vitro* methods are very often used which, however, fail to produce equally convincing results when tested in *in vivo* experiments again. For example, *in vitro* experiments in Switzerland with amphotericin B, chlortetracycline and formalin seemed to indicate their equal potential for successful treatment of ichthyophthiriosis as that of malachite green. *In vivo* tests, however, failed to corroborate that (Wahli et al., 1993). No adequate replacement for malachite green for the treatment of ichthyophthiriosis has been found. This is mainly due to the fact that these parasites penetrate into topical tissues of the body, and are thus partly protected against antiparasitic baths (Lom and Dykova, 1992).

The preparation Virkon S is also used as a malachite green substitute. It is a disinfectant (Antec International) patented in over 20 countries of the world. Virkon S is a blend of peroxygen compounds, surfactants, organic acids and inorganic salts that control the system pH. Its action mechanism is based on a strong oxidation effect on viruses, bac-

teria and fungi. For disinfection in aquaculture, 1:100 dilution (1% solution) is used and, according to currently available information, it is used in various European countries against a broad range of pathogens (<http://www.anticint.co.uk>).

5. Conclusions

In spite of partial success in the treatment of some diseases using the above “replacement” preparations, no truly adequate substitute for malachite green has been found.

Success in farming the economically important fish species hinges on a possibility of providing an adequate supply of good-quality water with minimum contamination by organic substances and on the use of good-quality feeds that will keep fish in the best health condition possible and increase their resistance to infections.

Because malachite green will persist in the aquatic environment for a long time and may pass via the food chain from there to untreated fish intended for human consumption, every care must be taken when malachite green used for baths of aquarium or ornamental fish is being disposed of. If enough attention is not paid to the problem, malachite green contained in baths or industrial waste water might penetrate to the aquatic environment and cause serious problems there.

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