MALACHITE GREEN

First draft prepared by
Dieter Arnold, Berlin, Germany
Bruno LeBizec, Nantes, France
and
Richard Ellis, Myrtle Beach, USA

IDENTITY AND PROPERTIES

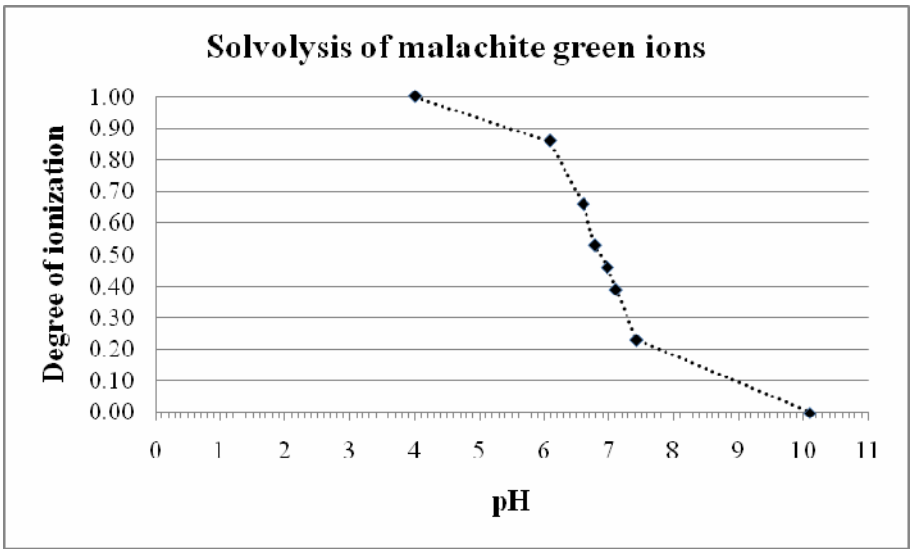
The structural identity, some major physical-chemical properties and molecular characteristics of malachite green salts, its carbinol base and its major metabolite leucomalachite green are summarised in Table 1. Other important properties are briefly summarised in the below subsections.

Chemical properties

Solvolysis

Goldacre and Phillips (1949) investigated the solvolytic reaction of malachite green and the formation of the carbinol at various pH values. For 2.7 x 10^{-4} M solutions at 25°C they found the following degrees of ionization shown in figure 1.

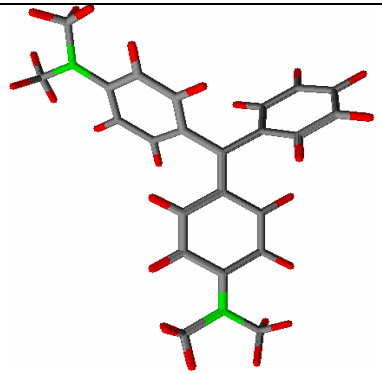
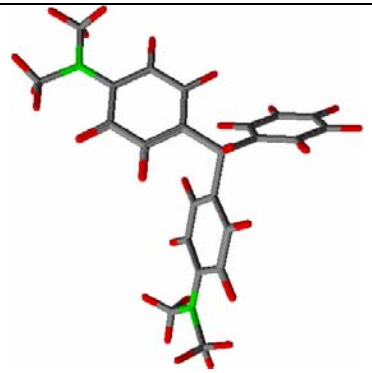
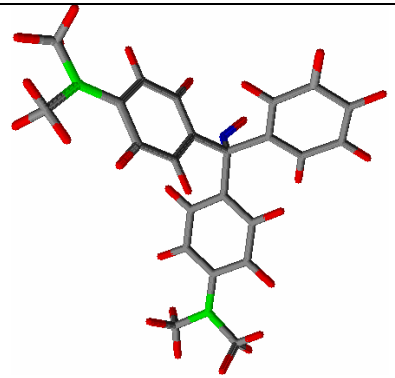
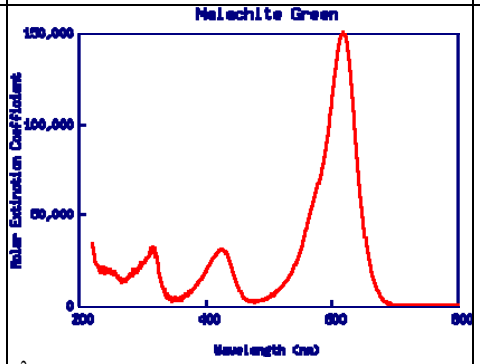
Figure 1: Solvolysis of malachite green as function of pH



From these results a pK of 6.9 was calculated. At this pH the time to decline half-way from 100% ionization to the equilibrium value is 2.1 hours. Velocity constants for the solvolytic reaction increase with increasing pH. The carbinol base which is less soluble than the ionized form is the form in which malachite green is probably taken up by fish. Therefore, the solvolytic equilibrium influences the pharmacokinetics of bath treatments.

Table 1: Physical-chemical properties of malachite green, its carbinol base and its metabolite leucomalachite green.

Substance name	Malachite green	Leucomalachite green	Malachite green carbinol base
Depositor-supplied synonyms (PubChem)	Total number: 82 Examples: Methylene green Grenoble Green Victoria Green Aniline Green Benzal Green China Green Fast Green Burma Green B Diamond Green Bx	Total number: 38 Examples: Leuco malachite green Malachite green leuco Malachite green leuco base Tetramethyldiaminotriphenylmethane C.I. Basic Green 4, leuco base	Total number: 20 Examples: Solvent Green- 1 Malachite Green carbinol Malachite Green Carbinol base
Chemical Abstracts Registry number	10309-95-2 (chloride: 569-64-2) (oxalate: 2437-29-8)	129-73-7	510-13-4
PubChem-CID	11295	67215	10521
EINECS	209-322-8 (chloride) 219-441-7 (oxalate)		208-109-7
IUPAC	[4-[(4-dimethylaminophenyl)-phenylmethylidene]-1-cyclohexa-2,5-dienylidene]-dimethylazanium	4-[(4-dimethylaminophenyl)-phenylmethyl]-N,N-dimethylaniline	bis(4-dimethylaminophenyl)-phenylmethanol
Molecular formula	$C_{23}H_{25}N_2+$	$C_{23}H_{26}N_2$	$C_{23}H_{26}N_2O$
Formula weight	329.46	330.47	346.47

Structure (propeller-like) prepared with Chemsketch 10.0			
UV-VIS spectrum	 λ_{max} is 616.5 nm H. Du, R. A. Fuh, J. Li, A. Corkan, J. S. Lindsey. PhotochemCAD: A computer-aided design and research tool in photochemistry. *Photochemistry and Photobiology*, 68, 141-142, 1998.	Not found	Not found
IR spectrum	Not found	http://webbook.nist.gov/cgi/cbook.cgi?ID=C129737&Units=SI&Mask=80#IR-Spec	Not found
Melting point [°C]	164	About 100	112.0 - 114.0

	(MG oxalate; Mallinckrodt-Baker 2006, http://www.jtbaker.com/msds/englishhtml/m0286.htm)		(Sigma-Aldrich 2006)
Octanol/water partition coefficient: logK_{ow}	0.62 (MG chloride)	Not found	Not found
Solubility in Water	40 g/l at 25°C (MG chloride) Baughman, G.L., et al; Dye Solubilities in: Advances in Color Chemistry, Freeman, M., Peters, M.T., eds., NY, NY: Elsevier (1993)	No information available	Low, exact data not found
Impurities found in commercial products[1]	Leucomalachite green, carbinol and demethylated derivatives and 4-DMABP	Malachite green and mono-desmethyl leucomalachite green	

Malachite green is typically available as hydrochloride or oxalate salt. The hydrochloride may have been obtained through precipitation with Zinc chloride and may be highly toxic to fish.

[1]See for example LeGoff and Wood (2008)

Other physical-chemical properties

Adsorption

Adsorption characteristics of malachite green were studied in order to find ways to remove it from waste waters. Traditional methods of wastewater cleaning only partly remove synthetic dyes such as, malachite green and structurally related compounds. The adsorption on a variety of inexpensive and more or less efficient inorganic and organic solid supports was tested as a method for the removal of malachite green from water and wastewater. When testing the adsorption characteristics of malachite green from aqueous solutions frequently only the kinetics of decolourisation of the aqueous solution was measured. Garg, et al. (2003) studied the properties of chemically treated saw dusts (e.g., formaldehyde, sulphuric acid). Gong, et al. (2006) studied citric acid modified rice straw and soybean hulls esterified with phosphoric acid. Hameed and El-Khaiary (2008) used rice straw-derived char. Mittal (2006) determined the adsorption kinetics from waste water onto hen feathers. Janos, et al. (2005) studied the sorption of malachite green from waters onto a naturally occurring kind of weathered and oxidized young brown, acid stable coal called oxihumolite. Tahir and Rauf (2006) used bentonite clay for the removal of malachite green from aqueous solutions. Wang and Ariyanto (2007) studied adsorption of malachite green to natural zeoliths.

Carbon-based sorbents show excellent adsorption properties for a considerable number of synthetic dyes. The preparation of carbon sorbents is generally energy consuming and large amount of carbon sorbent is needed for the removal of dyes from large volumes of water. Consequently, according to Forgacs, et al. (2004), the use of commercially available products is fairly expensive. Activated carbon can remove malachite green from water. A system for removal of malachite green from waters used for antifungal treatment in hatcheries was developed and described by Marking, et al. (1989). The removal efficiency was significantly less than 100%. For solutions containing 2 mg/l of malachite green an average of 23.4 mg of malachite green were adsorbed per gram of carbon.

Recent studies on the sorption using activated carbon with BET surface areas in the order of 1000 m^2/g prepared from different sources of material using various models for the calculation of adsorption isotherms (linearised and non-linear) and for studying the kinetics of adsorption. According to some authors the adsorption of malachite green was best described using a pseudo-second-order model with intra-particle diffusion of malachite green molecules within the carbon particles as a rate-limiting step. The following materials (examples only) were used for the preparation of activated carbon:

Authors	Material used
Singh and Rastogi (2004)	Used tea leaves
Rahman, et al. (2005)	Rice husks
Başar (2006)	Waste apricot
Onal (2006)	Waste apricot
Onal, et al. (2006, 2007)	Lignite,
Malik, et al. (2007)	Groundnut shell waste.
Kumar (2006), Kumar and Sivanesan (2006)	No information on source material
Porkodi and Kumar (2007)	Jute fiber.
Zhang, et al. (2008)	Arundo donax root

Several groups determined thermodynamic parameters (ΔH, ΔG, and ΔS) and concluded that the reaction was endothermic.

Malachite green is markedly biosorbed by activated sludge and reduces the rate of oxygen uptake by activated sludge proportionally to its biosorption (Mihara, et al., 2005). The results of the above studies suggest that malachite green and its metabolites and breakdown products may not be

completely removed by wastewater treatment and may be present in sufficient amounts in effluents from industry or waste water treatment plants or other sources to cause residues in wild fish. A group of researchers has recently claimed – without providing convincing evidence - that they have published the first example where malachite green was demonstrably taken up by eels caught downstream from treatment plants in the lakes and rivers surrounding Berlin, Germany (Schuetze, et al., 2008).

Photodegradation

Hydrogen peroxide has been frequently applied to decolorize synthetic dyes including malachite green in waters in the presence of suitable photo catalysts. For example, micro porous solid material prepared by precipitation of phosphotungstic acid and potassium ions, followed by calcinations was proposed for this purpose (Chen, et al., 2006). The formation of active oxygen species such as the radicals O_2^-, HO_2 and OH are detected during the degradation of dye, and they are proposed to be responsible for the degradation of dyes. In such systems CO_2 and small organic acids are the main reaction products.

The presence of catalysts such as TiO_2 enhances the rate of photodecomposition of malachite green under visible light. Both the superoxide anionic radical and the dye cationic radical are essential to the mineralization of the dyes under visible light-induced photo catalytic conditions (Arpaç, et al., 2007). Kominami, et al. (2003) used TiO_2 nano-particles with various physical properties that had been prepared by hydrothermal crystallization in organic media (HyCOM) and post-calcination, for photo catalytic decomposition of malachite green in an aqueous suspension under aerated conditions. Adsorptivity is a decisive factor for the initial bleaching of malachite green on this material which follows pseudo zero-order kinetics. Chen, et al. (2007) have studied the reaction mechanism of malachite green photo degradation on TiO_2. They identified a series of N-demethylated intermediates (mono-, di-, tri-, and tetra-demethylated malchite green) under basic reaction conditions of the process. These degradation products are also known to be formed metabolically in bacteria and animals (see below). Under acidic conditions, the whole conjugated chromophore structure of malachite green was cleaved (Chen, et al., 2007). Hydrogen peroxide can effectively decolorize dye wastewaters in the presence of Fe(III) – loaded ion exchange resin (Amberlite IRA 200). The degradation process of Malachite green proceeds via demethylation and phenyl ring openings before CO_2 and small molecules are formed.

Binding to macromolecules

Malachite green can bind to macromolecules. Of interest is the binding to small artificial RNA molecules (aptamers). The complex can exhibit interesting new properties, for example enzymatic activities (Brackett and Dieckmann, 2006). In the binding process the RNA adapts to the ligand (“adaptive binding”), but the ligand itself also undergoes conformational changes ("induced fit") (Nguyen, et al., 2002; Nguyen et al., 2004). The crystal structure of such complexes has been studied using tetramethylrosamine (TMR), a high-affinity analogue of malachite green (Baugh, et al., 2000). The properties and a number of applications of such complexes have been published. One possible use is the determination of malachite green itself, because aptamers are known which enhance malachite green fluorescence by factors in the order > 1000.

It is long known that malachite green binds to DNA (Nordén, et al., 1978; Bhasikuttan, et al., 2007). Cationic triarylmethane dyes also have complex-forming properties with proteins (Tacal and Ozer, 2004). A full discussion of all these properties is beyond the scope of this monograph.

Industrial uses of malachite green

Malachite green is used extensively as a dye for leather, wool, cotton, jute, paper, certain fibres, etc. For such purposes it has been produced in large quantities and extremely variable qualities. About 10-

15% of all dyes are directly lost to wastewater in the dyeing process (Parshetti, et al., 2006). Frequently the purity of products used in biological studies has not been reported. In human medicine the carbinol is/has been used as a wound antiseptic and as a treatment of mycotic skin infections, and in staining of tissues and bacteria.

RESIDUES IN FOOD FROM AQUATIC SPECIES AND THEIR EVALUATION

Conditions of use in aquatic animals

Malachite green has been used as a fish fungicide in closed systems alone or in combination with other chemicals such as formaldehyde for decades. Frequently reported concentrations are about 0.05 - 0.1 mg/kg. It is important to use zinc free preparations in order to avoid metal intoxications of the fish. Therefore, the oxalate was most frequently used. Foster and Woodbury (1936) were reportedly the first to introduce its use as fungicide and antiseptic. It took almost 47 years until researchers considered for the first time the possibility that malachite green could be taken up by fish (Poe and Wilson, 1983).

Malachite green has been used for the treatment of eggs of fish and crayfish. Malachite green is also an effective topical and systemic antiprotozoal agent. Reported types of treatment of fish include dip treatment, flush treatment, sustained culture treatment and application in feed. Extremely wide ranges of concentrations and exposure times have been used (review by Alderman 1985).

In a review of historical uses of malachite green, Sudova, et al. (2007) discriminate between dip treatments of 10-30 seconds duration and concentrations up to 100 mg/L to treat topical fungal infections, short-term malachite green bath treatments of 60-90 minutes duration at 6.7 mg/L, and long-term bath treatments of six days duration at 0.15 mg/L for salmonids and 0.5 mg/l for cyprinids. They state that this type of treatment was used to control protozoan ectoparasites, particularly the ciliated protozoan *Ichthyophthirius multifilii*s. Malachite green concentrations can be reduced in multi-component baths, for example in combination with formaldehyde. Treatment with malachite green can produce numerous side-effects in treated fish and fish eggs.

An important factor determining therapeutic and toxic effects is the temperature. Batch to batch variation in concentration and purity of the dye and lack of standardization of test conditions have been major confounding factors in the judgment of effectiveness of doses and exposure times. Therefore, it goes beyond the scope of this monograph to make any conclusive statements and comparisons about dosages and other conditions of use. Information on treatment conditions will be given individually in connection with the discussion of pharmacokinetic and residue studies.

Malachite green is toxic to fish, in particular to small fry. Bills, et al. (1977) used standard laboratory tests in order to determine the LC_{50} under various conditions of temperature, pH, and hardness of the treatment bath and of duration of treatment. Fingerling fish of a great variety of species, weighing 0.5 to 1.5g were used for the tests. Increase in exposure time significantly increased the acute toxicity. In short term-exposure (3 and 6 hours) of rainbow trout and channel catfish higher temperatures increased the acute toxicity. At the longest tested exposure time (96 hours) the temperature effect disappeared in rainbow trout. pH and hardness had no significant influence on acute toxicity. As an example, some data obtained with rainbow trout were selected from the original paper and are presented below in Table 2. The original work also provides the 95% conficence intervals of the LC_{50} which is not given in the below Table 2.

Table 2: Acute toxicity of malachite green to rainbow trout

Temperature [°C]	Water hardness	pH	Incubation time [hours]			
			3	6	24	96
			LC_{50} [mg/L			
7	soft	7.5	>2	2.3	0.4	0.17
12	soft	6.5	>2	1.0	0.28	0.28
12	soft	7.5	1.4	0.8	0.36	0.25
12	very soft	8	2.0	0.8	0.36	0.29
12	soft	8	2.3	0.8	0.28	0.23
12	hard	8	2.3	1.4	0.35	0.29
12	very hard	8	2.4	0.8	0.28	0.25
12	soft	8.5	2.6	1.0	0.28	0.21
12	soft	9.5	>2	1.3	0.37	0.17
17	soft	7.5	1.4	0.6	0.57	0.28

PHARMACOKINETICS AND METABOLISM

Pharmacokinetics in Fish

Physiological facts: Relationship between carcass weight and organ weight of fish

Some basic physiological facts are necessary to understand the kinetic behavior of malachite green in fish. Schmelzing and Claus (1990) found that in rainbow trout (*Oncorhynchus mykiss*) absolute organ weights increased with increasing carcass weight while their weights as a proportion of carcass weight decreased. Heart and liver weight were highly correlated with carcass weight, while the correlation between spleen and carcass weight was moderate. According to Corti (1948), muscle meat, liver and kidney make up 70.2, 1.2, and 0.77% of body weight in rainbow trout. The range of body weights (n=7) was 54.1 to 82.3g and corresponding range of muscle meat weight ranged from 61% to 78% of the body weights of the animals. The corresponding figures for eel were 80.9, 1.32, and 0.75 % for muscle meat, liver and kidney, respectively.

Fish physiology: Uptake of hydrophobic compounds

Fish normally take up hydrophobic compounds via the gills by passive diffusion (Gobas, et al., 1986). Rates of uptake can be a function of: water flow over the gills, blood flow through the gills, diffusion through the aqueous stagnant layer along the gill epithelium, or diffusion through the gill membrane. Hayton and Barron (1990) have summarized: "In general, for any particular chemical, only one of the barriers is operative with the resistance offered by the others being negligible. The rate-limiting barrier is determined by the physico- and biochemical properties of the substance: molecular size, lipophilicity, binding to blood proteins and formed elements. The resistance of each barrier is affected differently by variables such as temperature, molecular size, lipophilicity and body size of the animal. When the resistance offered by the gill barriers is low, uptake may be controlled by transfer to storage tissues, e.g., by blood flow to adipose tissue".

When fish increase their water flow, e.g., with decreasing oxygen concentrations or other types of stress, uptake per unit time frequently increases. Sijm, et al. (1994) studied the influence of blood and water flows on the uptake of some hydrophobic compounds by rainbow trout. The fish used in their experiments had an average weight of 110 ± 12g (n not given). The temperature was 12°C. For all compounds studied the uptake rate constants increased with water flow between 0.045 and 0.52 l/min/kg body weight and remained constant at higher flow. The uptake rate constant was constant for blood flow between 4.4 and 10 ml/min/kg body weight but doubled when the blood flow was

increased from 10 to 20 ml/min/kg body weight. From their findings they deduce that water flow will practically limit uptake of hydrophobic chemicals in fish weighing more than 5g.

Gill physiology

In a study on water flow and gas exchange at the gills of rainbow trout (*Salmo Gairdneri*) Erickson and McKim (1990) developed a simple flow limited model for the exchange of organic chemicals at fish gills. The mathematical model for the exchange of organic chemicals by fish gills was formulated based solely on the limitations imposed by the flows of water and blood into the gills. The model could be useful for approximate assessments of accumulation of organic chemicals by fish. For large rainbow trout, the model was found to closely follow the magnitude and trends of observed gill uptake rates over a range of octanol/water partition coefficient from 1 to 10^6.

Davis and Cameron (1971) estimated the volume of water passing over the gills per unit time (ventilation volume). The technique was direct measurement. For this purpose a rubber membrane was stitched round the margin of the mouth of the fish in a way that it separated inspired and expired water. 18 fish of a body weight of 210.3 ± 2.3 g were used at 8.6°C to determine ventilation volume when the animals were quiet. The estimation was repeated 4-11 times with each animal. The lowest individual estimate of ventilation volume observed in one fish was 22.0 ml/min; the average per fish ranged from 26.0 to 49.0 ml/min. The overall average was 37.0 ±7.4 ml/min. The corresponding mean ventilation rate was 74 breaths/min and the mean ventilator stroke volume was 0.5 ml/breath. When the fish struggled or were disturbed maximum values rose as high as 162 ml/min in one animal (average 88.2 ± 43.7).

Nichols, et al. (2004) developed a physiologically based toxico-kinetic model for dietary uptake of hydrophobic organic compounds. Malachite green administered via the diet shows unsatisfactory efficacy. Therefore, this model cannot be applied to the data discussed in this monograph.

Pharmacokinetic studies in rainbow trout

Alderman and Clifton-Hadley (1993) conducted a pharmacokinetic study in rainbow trout (*Onkorhynchus mykiss*). The dye was administered through uptake from the water bath. The heavily vascularized gill was assumed to be the principal site of malachite green uptake from solution under these conditions

The fish used in the main pharmacokinetic experiments of the study were two separate groups of rainbow trout with average body weights of 241 ± 33 g (n not given) for studies conducted at 16°C and 199 ± 20g (n not given) used for studies conducted at 8°C, respectively. A third group of 30 fish of 50g body weight was used for additional experiments in which residues in muscle were determined in individual fish at 16°C (results not given). The pH was 7.6 and total hardness was 13.8° dH. Under these conditions more than 95% of the malachite green was in the carbinol form. The purity of the malachite green was tested by thin layer chromatography. Treatment solutions containing 1.6 mg/l (4.86 µmoles/l) were prepared from a commercial liquid formulation 15 h before use in order that the dye-carbinol equilibrium concentrations could become established. Treatment time was 40 minutes.

In the main experiments fluids and tissues of five fish per time point were pooled and stored frozen for analysis. However, one graph of the publication shows individual kinetic data in serum obtained in a separate experiment. Samples of serum and bile were allowed to defrost before analysis. After dilution with buffer at pH 4.0 they were extracted for 24 h into pentan-1-ol (no information on the partitioning of malachite green and its metabolites was provided). After 24 h, samples were centrifuged at 2000 × g for 30 min, giving a clear pentan-1-ol supernatant. All other tissues were allowed to defrost overnight and weighted composites were then blended in 2% pepsin adjusted to pH 2.0 with HCl. The blended samples were kept for 18h at room temperature (20°C) and were shaken thoroughly several times in that time. The samples were then partitioned at room temperature at approximately pH 4 into pentan-1-ol. Following the addition of pentan-1-ol the flasks were shaken

vigorously before being left (with further shaking) for a further 24 h. Samples were then shaken thoroughly again before centrifugation at 2000 × g for 30 min at 4°C.

Sample extracts were scanned at wave lengths from 540 to 700 nm. Peak absorption for the malachite green dye ion in extracts was 625 nm. Reported recoveries at 10 mg/kg were 19, 82, 75, 68, and 60% for serum, liver, kidney, muscle, and viscera, respectively. The spectrophotometer was calibrated against extracts of malachite green obtained from representative fish tissues spiked with known concentrations of malachite green and after equilibration subjected to the same extraction procedure as described above for the experimental samples. The reported LOD was about 50 µg/kg (data not shown). Some tissues presented general or occasional problems in residue determination including interference of colored co-extracted substances. The paper exhibits a number of weaknesses:

- Concentration of the drug in water was not monitored during the experiment.
- The description of the experiment lacks precision. The exact relationship between total weight of treated fish and weight of the bath is not given. One may speculate that it not exceeded 24 x 5 x 0.241 kg of fish in 725 kg bath.
- Fat was not sampled.
- It was not determined to what extent leucomalachite green was extracted and whether it was re-oxidized to the malachite green. Therefore, the kinetic data of this study can probably not be interpreted.
- Insufficient information on method validation is provided. The study typically does not provide information on results obtained with individual animals; thus no estimate of the biological variability is possible.
- The authors report that malachite green appeared in the serum very rapidly, with concentrations increasing steadily until the fish were removed from the dye (results not shown). In fact, peak concentrations shown in figure 2 of the paper were in the order of 13-13.5 mg/kg at both 8 and 16°C.
- The text states that peak concentrations in muscle were reached 90-120 min after the end of exposure and gives values of 6.81 and 10.79 mg/kg for the peak concentrations reached at 8°C and 16°C, respectively; however, the curve describing the influence of exposure time never exceeds approximately 1 mg/kg for muscle.
- The text states that when a group of small rainbow trout was exposed to malachite green and individual muscle residues examined at 24 h post-exposure, considerable fish-to-fish variations were evident; however neither data nor an estimate of the variance are provided.
- The legend to the figure describing the kinetics in bile and the corresponding text state that bile could only be reliably collected for the first 40 h; however, the corresponding graph shows data points for 48 and 72 hours.
- The analytical method used was inadequate.
- The calculated half-lives for serum and tissues cannot be compared because they are either based on different kinetic models used for curve fitting, or - even when the same model was used - the distribution in time of the data points covered different phases of the kinetics.
- Some variations in treatment parameters and associated effects on results are discussed without providing any data.
- Extrapolations (calculations and results not shown) far beyond the experimental time points and orders of magnitude below the measured concentrations.

The following conclusions may be drawn from the results of the study: Uptake was lower at 8°C than at 16°C; the initial rate of decrease of the optical density at 625 nm was higher at 16°C than at 8°C; the maximum concentrations found in tissues were in the order of (all values in mg/kg):

Temperature/Tissue	Serum	Liver	Kidney	Muscle
8 °C	13		8	7.8
16 °C	13.5	16.5	34	10.8

However, this statement is only valid if one can assume that the biotransformation of malachite green in this experiment was a slow process compared to the rate of uptake or that leucomalachite green is not picked up by the analytical method. If one assumes that malachite green was rapidly metabolized to leucomalachite green and other molecules (as shown in other studies) which are all extracted, then all results of this study could be meaningless numbers; if leucomalachite green was re-oxidized the above given maximum concentrations could represent an estimate of total residue.

A table summarizing pharmacokinetic and residue data in aquatic species was found on the website of the U.S. FDA. The table includes the work of Alborali, et al., published under the title: "The persistence of malachite green in the edible tissue of rainbow trout (*Oncorhynchus mykiss*)" in the Journal Rivista Italiana di Acquacoltura 32, 45-60. The summaries of the cited findings are given here: Rainbow trout (*Oncorhynchus mykiss*) with a body weight of 60-80 g were exposed at 18 °C for one hour to a solution of 1 mg/l of malachite green. Residues were determined by HPLC (no details given). The following information contained in the FDA document is provided for gills, kidney, muscle and skin:

"Gill: Residues decreased fairly rapidly during the first 320 days reaching 260 by d41, and below 1 ng/g after 7th month. T 1/2 in the 20 d range initially, with later slow decline in the 50d range.
Kidney: Max residue on d(ay) 30 = 1650 ng/g, declined to below 1 ng/g after 41 d.
Muscle*:* Residues high for 34 days - above 1,000 ng/g. Decreased to 200 ng at the 4th month, around 100 by 150 d, slowly declining to below 10 ng/g by 9th month. $T_{1/2}$ in the 40-50 d range.
Skin: Residues decreased fairly rapidly during first 20 days, to about 2500 ng/g, d 50= approx 1000, 200 ng/g round the 4th month, below 1 ng/g at day 283. $T_{1/2}$ in the 50 d range."

Pharmacokinetic studies in channel catfish

The pharmacokinetics and metabolism of malachite green in channel catfish (*Ictalurus punctatus*) were examined by Plakas, et al. (1996) after intravascular dosing or waterborne exposure. The intravascular dosing solution contained 0.8 mg of ^{14}C labeled dye cation per ml of 0.85% aqueous NaCl solution corresponding to a specific activity of 0.925 MBq/ml or 1.16 MBq/mg. For waterborne exposures, the initial dye concentration was 0.8 mg/l corresponding to a specific activity of 0.185 MBq/ml or 0.231 MBq/mg. The channel catfish were 0.5 to 0.7 kg. For the collection of blood and urine the dorsal aorta and urinary bladder were cannulated.

- Five animals were dosed intravascularly with [^{14}C] malachite green at a dosage of 0.8 mg/kg body weight. Blood specimens were collected 2.5, 5, 7.5, 10, 15, 20, 30, and 45 min and 1, 2, 4, 6, 8, and 10 h after drug administration.
- Five animals were transferred to the dosing solution (0.8 mg/l). Blood specimens were taken at 15-min intervals during the 1-h exposure period. At the end of the dosing period, fish were briefly rinsed in a water bath. Blood specimens were taken at 10, 20, 30, and 45 min and at 1, 2, 4, 6, 8, and 10 h after the end of the dosing period.
- To determine the tissue distribution of malachite green and its residues after waterborne exposure, groups of five animals were exposed to [^{14}C]-malachite green solutions (0.8 mg/l) for 1 hour. Animals were killed and tissues collected immediately after dosing (designated 0 h) and at 2, 4, 24, 96, 168, and 336 h (14 days). Additional animals dosed with unlabelled malachite green were sacrificed after 28 and 42 days.

For HPLC determination of malachite green and leucomalachite green, plasma was extracted with acetonitrile. Muscle was subjected to a more complex procedure involving extraction with acetonitrile – acetate buffer, re-extraction, solvent partition, and SPE. HPLC fractions were subjected to post-column oxidation to the malachite green ion. Mean extraction efficiencies for malachite green and leucomalachite green from plasma and muscle were 85 - 95%. Mean recovery of total radioactivity from muscle of treated fish was 88%, however, individual animal data were not provided.

The mean of the concentrations of the plasma samples of five animals declined rapidly after intravascular dosing. Simultaneously the corresponding concentrations of leucomalachite green increased rapidly and reached an average maximum concentration of 0.875 µg/ml in the samples taken at 0.75 h after dosing. At this time point the corresponding concentration of the parent malachite green was 0.6 µg/ml. At ten hours the concentrations of leucomalachite green and of parent malachite green were 0.20 and 0.05 µg/ml, respectively. The sum of these two compounds accounted for approximately 70% of the total drug equivalents at each sampling time. The authors fitted a six parameter (three exponential terms) equation to the data obtained at 14 time points of which nine points were collected during the first hour after treatment. They estimated a terminal half life of 6.2 hours for malachite green.

During waterborne exposure at 21 ^{o}C total radioactivity and the concentrations of both malachite green and leucomalachite green increased very rapidly to 2.77 and 1.56 µg/ml plasma, respectively. Concentrations of malachite green then started declining immediately; however the peak concentration of leucomalachite green was 2.36 µg/ml 1 hour after transfer of the fish to clean water. The authors state that the decline followed a tri-exponential curve (results not shown) and estimated a terminal half life of 4.7 h. After 10 h concentrations of malachite green in plasma had declined to the limit of detection of 0.25 µg/ml. The authors state that the concentrations of leucomalachite green were still 30 times higher. The half life of terminal depletion of leucomalachite green was not estimated. No information on biological variability is available. The water bath conditions were such that the ratio of the ionic and the carbinol form of malachite green was 6:10.

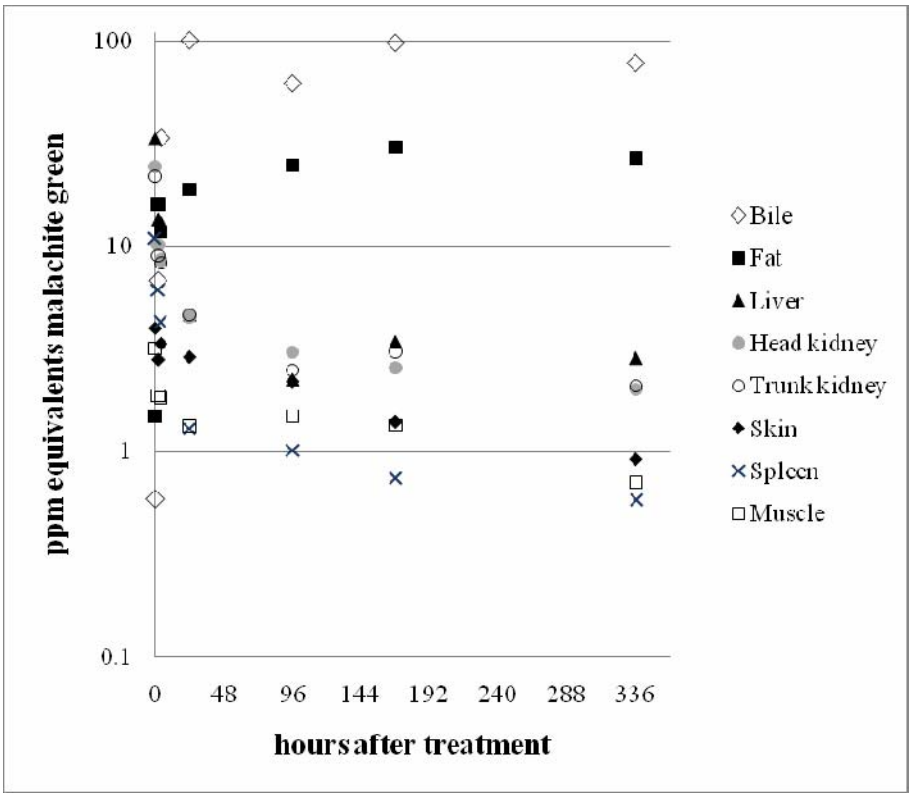
Malachite green and its metabolites were widely distributed and concentrated in the tissues. Concentrations of radioactive residues were highest in the excretory tissues and fat and lowest in the muscle and plasma. Concentrations of total residues exceeded the initial concentration of the water bath in all tissues, with the following few exceptions: bile (0 hours), spleen (168 and 336 hours), and muscle (336 hours). The authors' results, originally in tabular form, are summarized in figure 2 below. The variability of the results expressed as relative standard deviation of the mean of 5 data points ranged from 0.07 to 0.76. Variability was lowest in muscle tissue and highest in skin and bile. The variability increased over time. The high values of many standard deviations suggest that the residue concentrations might not be normally distributed and that the averages calculated by the authors are not the ideal parameters to show a central tendency. Concentrations of residues in skin were always higher than in muscle. The highest ratio was 2.2, observed 24 hours after treatment.

The concentrations of malachite green and of leucomalachite green in plasma were 3.29 and 1.94 µg/ml, respectively, immediately after dosing. One day after dosing, malachite green levels were at the LOQ while leucomalachite green levels were 0.11 µg/ml at day 14 after treatment.

In muscle, malachite green and leucomalachite green concentrations were 1.18 and 1.45 mg/kg, respectively, at the end of the exposure period and 0.012 and 0.52 mg/kg 14 days after treatment. Results obtained at other time points are not numerically given. The elimination of malachite green in muscle appeared biphasic with a terminal half-life of about 67 h. Concentrations of leucomalachite green were quantifiable for up to 42 days (0.02 mg/kg). Unidentified metabolites eluting before leucomalachite green during HPLC were found. The sum of the concentrations of these three metabolites reached a maximum of 31.3% of the total residue at 24 hours after treatment. No detailed pharmacokinetic information is provided for the water-borne exposure; however the authors state that the half lives for malachite green and for leucomalachite green were 2.8 and 10 days, respectively.

The effect of pH of the exposure solution was studied at pH values of 6, 7, and 8. When catfish were exposed to solutions of 0.8 mg/l of malachite green for one hour, uptakes increased significantly with increasing pH, determined by the concentrations of malachite green and leucomalachite green in plasma and muscle immediately after exposure. This may be due to the change in equilibrium concentrations of the cation and the carbinol and in the rates of conversion of the cation to the carbinol. The well designed and conducted study cannot be used for the derivation of MRLs because statistical evaluations are not possible in the absence of individual animal data.

Figure 2: Total residues of malachite green in tissues of channel catfish.



Pharmacokinetic studies in juvenile eels

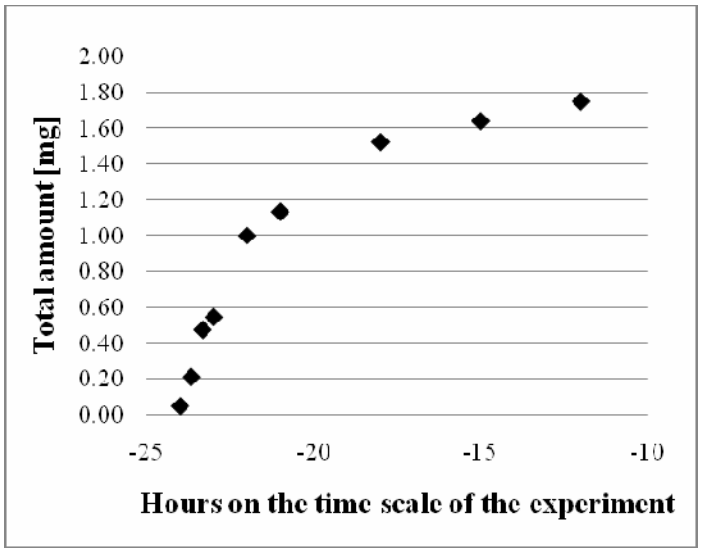
Bergwerf, et al. (2004) exposed 450 juvenile eels (*Anguilla anguilla*) of an average weight of 4.1 g (range 1.5 to 7.4 g) for 24 hours to malachite green in a water bath with temperature varying between 23.0 and 26.5°C and at pH values between 7.0 and 7.8 in 40 L water. A malachite green concentration of 0.1 mg/l was intended. Fish were then cultured in malachite green free water at 21.6 to 27.5°C. During this period pH varied between 6.0 and 7.8. Before, and at various time points during, and up to 100 days after treatment, ten fish and water were sampled (50 ml per time point). The fish did not grow during the study.

The whole fish were cut in fine pieces and 2g of cut tissues were blended in buffer and extracted with buffer/acetonitrile mixtures. Partitioning with dichloromethane was followed by solid phase extraction. Brilliant green was added as internal standard before HPLC analysis. Water samples were mixed with buffer, acetonitrile and internal standard prior to analysis. Two reversed phase columns (phenyl-hexyl and C_8) were used in series and were eluted with a mixture of 60% (vol/vol) acetonitrile and 40% (vol/vol) of 0.05M ammonium acetate buffer, pH 4.5, at 0.6 ml/min. The eluate was monitored at 620 nm after post column oxidation. Recoveries (n=36) were 61 ± 6% for malachite green and 88 ± 10% for leucomalachite green. Results were recovery-corrected.

Analysis of water revealed that the starting bath concentrations were only 0.032 mg/L instead of 0.1 mg/l. This concentration further decreased exponentially during the experiment and fell below the limit of detection at 12 hours. A definitive reason for the low initial concentration could not be found. The further decrease can apparently be explained by the uptake of malachite green by the fish. One can roughly estimate the total amount of malachite green taken up by the fish from the sum of the concentrations of malachite green and leucomalachite green found at a given time point multiplied with number and average weight of fish present in the bath at this time point plus the accumulated amounts removed from the bath by sampling 10 fish at every earlier time point. The figure 3 shows

the results of such a crude calculation. The total amount of drug initially present in the bath was estimated as approximately 1.28 mg using the information given by the authors. The small discrepancies are probably due to the crude estimates used in these calculations.

Figure 3: Estimated time course of the exhaustive uptake of malachite green in the exposure experiment.



The density of fish in the small bath which absorbed the drug and the long exposure time probably caused a total uptake of the malachite green present. Even the higher originally intended concentration would have been too low for such an experiment. The figures 4a and 4b summarize the pharmacokinetic results of the experiment. The symbols represent mean values. The bars show the range. If a lower bar extends down to 0.2 µg/kg, the result of the analysis was <LOD. The LOD was given as 0.2 µg/kg.

Figure 4a: Concentration changes of malachite green and leucomalachite green during and after bath exposure to 0.032 mg/L of malachite green.

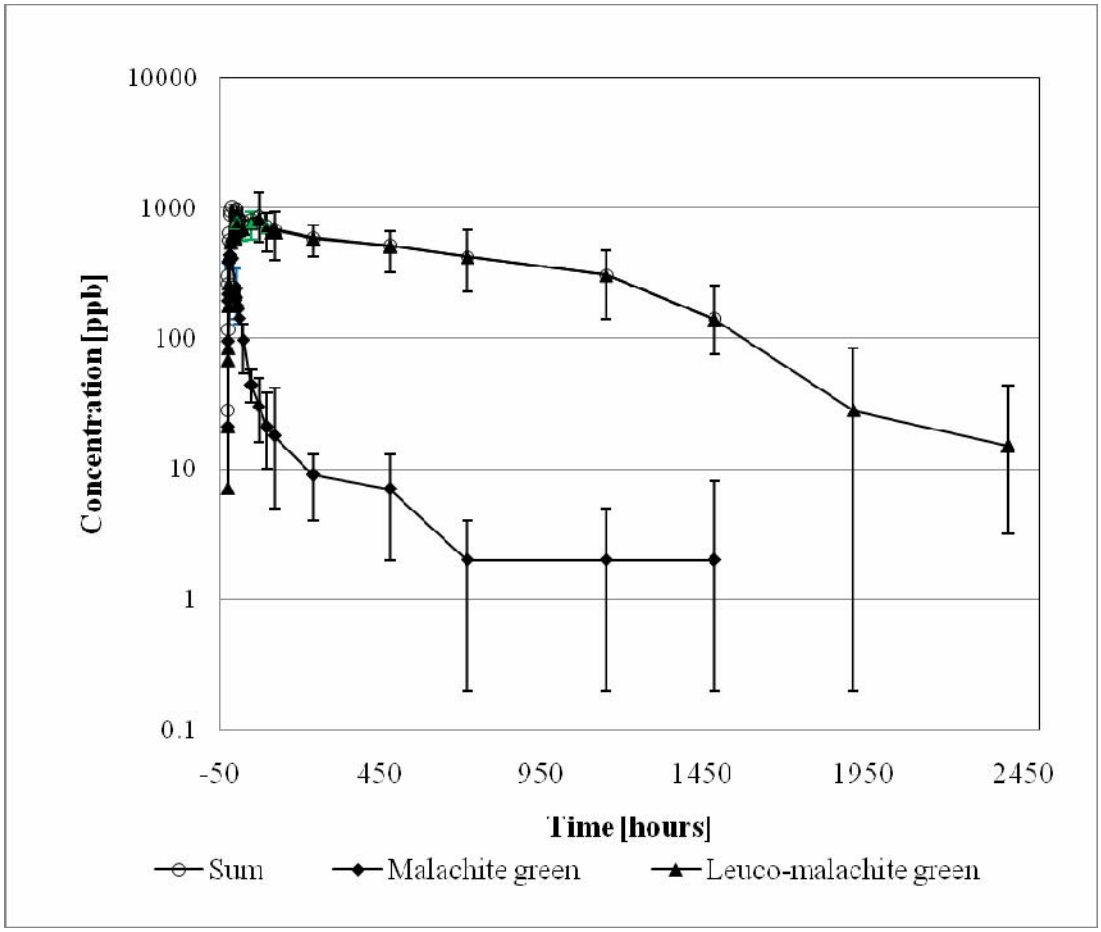
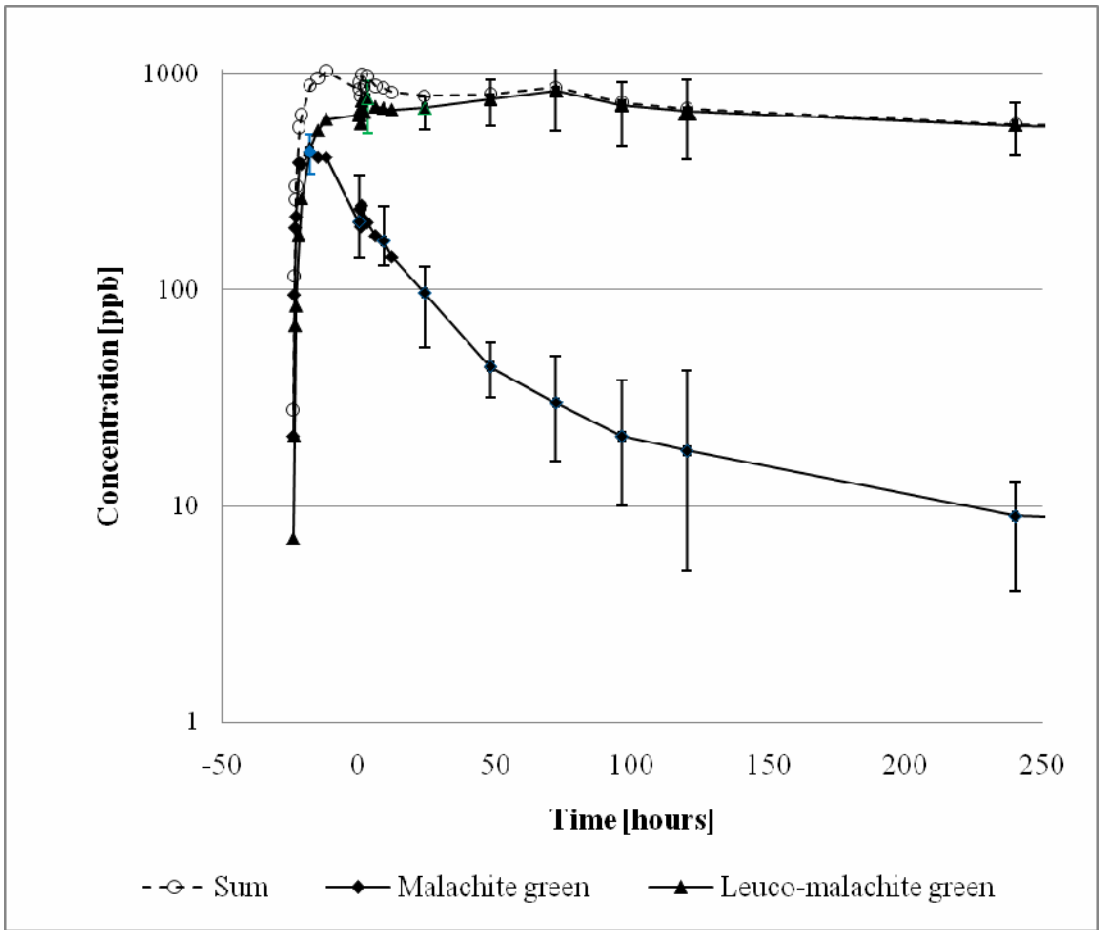


Figure 4a shows the averages of the measurements obtained with 9-10 individual fish at every time point and the range of the individual values. It is not clear how the averages have been calculated at time points where "non-detects" occurred. The latest time point at which the results were not influenced by "non-detects" was 480 hours for malachite green and 1487 hours for leucomalachite green. Malachite green was quantified in the one or other fish up until day 62 following exposure and leucomalachite green was found in the one or other fish over the whole 100 days observation period following exposure. This finding is important in view of the very limited dose administered.

Figure 4b: The exposure and early depletion part of figure 4a.



For graphical reasons figure 4b shows bars indicating ranges of individual observations only for selected time points well spaced on the time scale. The figure shows that almost immediately with the uptake of malachite green at the beginning of the exposure period also the concentration of leucomalachite green starts increasing. Due to the circumstances described above, the data of this study are neither useful for pharmacokinetic evaluations nor any further interpretations.

METABOLISM

Metabolism in Micro-organisms

Fungi

Some ligninolytic fungi have been found capable of decolorizing synthetic dyes. This is due to their production of enzymes such as, laccase and Mn-peroxidase that enable these microorganisms to oxidize a broad range of substrates. Studies have focused on the possible use of some model wood-rotting white-rot species (*Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus* and others) for decolorization of synthetic dyes. Ligninolytic cultures of the white rot fungus *Phanerochaete chrysosporium* were shown to metabolize crystal violet and malachite green to N-demethylated metabolites catalyzed by lignin peroxidase. Extracellular fluid obtained from ligninolytic cultures of this fungus, retained the activity provided that an H_2O_2-generating system was supplied. Non-ligninolytic (nitrogen-sufficient) cultures also degrade crystal violet by another mechanism without producing N-demethylated metabolites (Bumpus and Brock, 1988). Eichlerova, et al. (2006a) investigated the dye decolorization capacity of two white rot fungi, *Dichomitus squalens*

and *Ischnoderma resinosum* (Eichlerova, et al., 2006b). *D. squalens* showed high decolorizing capacity. *I. resinosum* decolorized malachite green to a lower extent up to a concentration of 0.1 g/l.

A total of 26 white rot fungi from Argentina were tested for their ability to produce lignin-modifying enzymes and decolorize industrial dyes (Levin, et al., 2004). Ten of the strains decolourised all tested dyes including malachite green. The mycelia were grown on solid malt extract/glucose media containing the dye. All ten strains produced laccase, lignin peroxidase and manganese peroxidase on solid medium. White-rot fungi normally require a lignocellulose substrate. Their use in polluted water streams or soils may be problematic since species of these typical wood colonizers do not exhibit satisfactory growth and competitiveness under such conditions.

Litter-decomposing fungi differ from wood-rotting species with respect to their growth substrate, forest litter and soil. They are characterized by higher C:N ratio and microbial activity. Laccase is the most common ligninolytic enzyme among these organisms and Mn-peroxidase is produced only by some species (Baldrian and Snijdr, 2006).

Cha, et al. (2001) performed biotransformation experiments of malachite green with cultures of *Cunninghamella elegans*, a filamentous fungus which had previously been shown to enzymatically catalyse N-demethylation and N-oxidation reactions of a number of chemicals. Metabolites were analysed using HPLC-diode array and HPLC-MS methods. Malachite green was reduced to leucomalachite green and also converted to N-demethylated and N-oxidized metabolites, including primary and secondary arylamines. The mono-, di- and tri-desmethyl derivatives of malachite green and the mono-, di-, tri-, and tetra-desmethyl derivatives of leucomalachite green were found in the supernatant following removal of the mycelium. Malachite green N-oxide was only detected in the mycelia. Identical patterns of metabolites were observed with malachite green and with leucomalachite green as initial substrate. After prolonged incubation only reduced metabolites were found suggesting that parent malachite green and N-demethylated metabolites were reduced by the fungus. Microsomal fractions did not produce reduced metabolites in the absence of NADPH. The cytochrome P450 inhibitor metapyrone completely inhibited the biotransformation reactions.

Intestinal bacteria

Henderson, et al. (1997) studied the metabolism of malachite green by intestinal microflora from human, rat, mouse, and monkey fecal samples and 14 pure cultures of anaerobic bacteria representative of those found in the human gastrointestinal tract. All complete microfloras were very efficient in reducing malachite green to leucomalachite green (human and rhesus monkey intestinal microfloras, C3H/HEN-MTV mouse intestinal microflora, and Fisher 344 rat intestinal microflora). Of the bacteria commonly found in the human intestinal tract, *Clostridium perfringens (ATCC 3624)*, *Escherichia coli (ATCC 25922)*, and *Peptostreptococcus anaerobius (ATCC 27337)* converted almost all of the dye to the leuco derivative. The conversion was monitored with HPLC with diode array detection and the structure was confirmed by mass spectrometry.

Baker's yeast (*Saccharomyces cerevisiae (MTCC 463)* was also shown to effectively decolorize malachite green, primarily through reductive pathways (Jadhav and Goindwar, 2006). A number of other bacteria have been positively tested for decolorizing capacity of malachite green. A complete review would go beyond the scope of this monograph.

Metabolism in Laboratory Animals

Rats and mice

In short term feeding studies, Culp, et al. (1999) have shown that MG is sequentially *N*-demethylated to secondary and primary aromatic amines in rats and mice both before and after reduction to LMG. Female and male B6C3F_1 mice and Fischer 344 rats were fed up to 1200 mg/kg malachite green or 1160 mg/kg leucomalachite green for 28 days. The malachite green used was ≥ 94% pure. Impurities

detected were leucomalachite green (1%) and demethlyated derivatives of malachite green (3.5%). Leucomalachite green was ≥ 98% pure. Impurities detected were malachite green and mono-desmethyl leucomalachite green. Livers were extracted using a modification of a published method (Roybal, et al., 1995). The extracts were analysed by HPLC connected to a post-column oxidation chamber and a photodiode array detector. Analyses using HPLC-APCI/MS also were performed. The desmethyl derivatives were synthesized to confirm structures in the samples subjected to APCI/MS.

In HPLC-APC/MS analysis of liver extracts from rats treated with leucomalachite green the primarily seen compounds were protonated leucomalachite green, protonated demethylated derivatives and the molecular ions of malachite green N-oxide and demethylated N-oxide. A small, but measurable, amount of malachite green was also found. At higher cone voltages additional collision-induced diagnostic fragments were found that were formed following losses of dimethylaniline-, methyl-, or phenyl- moieties. The appearance of these molecules was consistent with the fragmentation pathways previously published for leucomalachite green (Doerge, et al., 1998a) who observed similar sequential demethylation in a thyroid peroxidase-catalyzed reaction of leucomalachite green. A dose-related increase in leucomalachite green and metabolites was observed in both rat and mouse liver extracts.

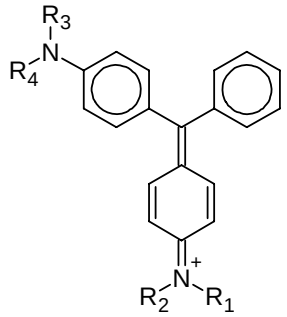
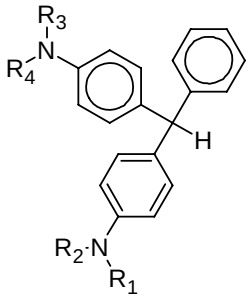
Similarly, HPLC-APC/MS analysis of liver extracts from rats treated with malachite green detected the molecular ions for malachite green, its mono-, di-, tri-, and tetra-desmethyl derivatives, and malachite green *N*-oxide. A small, but measurable, amount of leucomalachite green was also detected. Higher cone voltages produced fragments consistent with those previously reported by Doerge, et al., (1998b). These authors incubated leucomalachite green with tyrosin peroxidase, iodide, and tyrosine in the presence of an H_2O_2 generating system and obtained the mono-, di-, and tri—desmethyl derivatives of leucomalachite green as well as malachite green and malachite green-N-oxide. Concentrations of malachite green and metabolites increased with increasing dose.

The formation of both the symmetric and asymmetric di-desmethyl malachite green metabolite could be demonstrated with the symmetrical isomer eluting first. When liver extracts were analysed using HPLC/UV detection, leucomalachite green was the major product detected in rats fed leucomalachite green (accompanied by small amounts of mono- and di-desmethyl-leucomalachite green) and malachite green was the major product detected in the livers of rats and mice fed malachite green (accompanied by mono- and di-desmethyl malachite green and leucomalachite green – and in the case of rats mono- and di-desmethyl-leucomalachite green).

^{32}P-Postlabeling of liver DNA indicated the formation of a DNA adduct, or co-eluting adducts, that increased with increasing dose, in rats and mice fed leucomalachite green or malachite green. Cho, et al. (2003) mention that malachite green and the *N*-demethylated derivatives of malachite green and leucomalachite green are capable of forming DNA adducts *in vivo*, with the binding being consistently greater with the ionic MG derivatives.

Figure 5 below summarizes the structural elements of the N-desmethyl metabolites of malachite green and leucomalachite green.

Figure 5: Structures of malachite green and leucomalachite green.

Structures of malachite green, leucomalachite green, and demethylated derivatives								
	Malachite green				Leucomalachite green			
	R_1	R_2	R_3	R_4	R_1	R_2	R_3	R_4
Parent molecule	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3
Desmethyl-	CH_3	CH_3	CH_3	H	CH_3	CH_3	CH_3	H
Di-desmethyl- (symmmetric)	CH_3	H	CH_3	H	CH_3	H	CH_3	H
Tri-desmethyl-	CH_3	H	H	H	CH_3	H	H	H
Tetra-desmethyl-	H	H	H	H	H	H	H	H

Metabolism in Food Producing Animals

Fish

No systematic metabolism study has been performed in fish. However, many of the degradation products formed either under physical-chemical conditions or in *in vitro* and *in vivo* studies in bacteria, fungi and laboratory animals have also been found in fish. The potential presence of such molecules in monitoring samples is largely ignored by analysts and only a few methods use protective agents in order to prevent the breakdown of incurred residues during extraction and cleanup.

TISSUE RESIDUE DEPLETION STUDIES

Fish

Poe and Wilson (1983) reported in a short note to the journal "Progressive Fish Culturist" that they had observed a green color developing after some storage time on the surface of the tissue of skinned frozen fish. The color could be extracted using a procedure for lipid extraction and was identified as malachite green by infrared and visible spectra. They conducted a small series of experiments with channel catfish of body weights ranging from 0.3 to 0.7 kg in which they varied exposure conditions (exposure levels and frequencies). Samples of visceral fat and the carcass were frozen. The green color appeared typically in visceral fat more rapidly than in muscle. When Alderman published his frequently cited article "Malachite green: a review" in 1985, uptake and residues had not yet become a significant issue in the scientific literature.

Eggs and fry

Allen and Hunn (1986) reported that malachite green accumulates in the eggs of gravid female salmon after treatment and is detectable in eggs and newly hatched fry.

Meinertz, et al. (2005) determined residues of [^{14}C]-malachite green in eggs and fry of rainbow trout after treatment of the eggs. The treatment method was flush treatment (a concentrated solution of malachite green was added to an incubation unit and flushed through with fresh water). At the beginning of the treatment the eggs were approximately 2 days old. Hatching began on day 25 and

was completed on day 31. Six groups of 250 eggs each were treated in 500-ml glass test aquaria. Before treatment water flow to the aquaria was stopped and the volume was drawn down to 265 ml. Eggs in all aquaria were exposed simultaneously using a distribution manifold. The expected nominal concentration was 1.0 mg/l. Water flow was re-established immediately after treatment. Treatment was performed on days 0, 3, 6, 9, 12, 15, 18, 21, 24, and 31. Water samples were taken at every treatment from one randomly selected aquarium immediately after addition of the treatment solution and 2.5, 5, 10, 15, 20, 25, 30, and 60 minutes after water flow was re-established.

Ten eggs were sampled from each test aquarium immediately before each treatment through day 24. Five were prepared for combustion analysis (92.8 to 95.8 combustion efficiency) and the remaining five were used for analysis of malachite green residues by HPLC with post-column oxidation and visible light detection. On day 31, 10 fry were sampled from each aquarium immediately before the final treatment and 0, 6, 12, and 24 hours, and 2, 4, 7, 12, 17, 22, and 28 days after treatment. Mean peak concentrations of chromatic malachite green in water were 0.37 mg/l and were slightly higher than the radioactive concentration equivalents. The intended nominal concentration was not reached and individual measurements were extremely variable at all time points.

Untreated eggs contained measurable concentrations of unlabelled malachite green. Pre-treatment radioactive concentration equivalents in eggs and fry increased from day 0 to day 31 to a concentration of 271 ± 42 (n=6) – without exhibiting saturation effects or reaching a steady state - and declined to 55 ± 11 on day 28 after the final treatment. The efficiency of the analytical method to extract radioactivity was 49 to 119 % (average 76%). Leucomalachite green was the predominant residue. OD and radioactivity traces of HPLC separations showed one more polar unknown compound in addition to the known compounds. If measurements in fry were corrected for growth, the elimination half life was 9.7 days.

Kinetic depletion studies in fish

In addition to the studies described in the section of pharmacokinetics some other studies have been performed. Bauer, et al. (1988) published an article “Uptake and excretion of malachite green in rainbow trout”. They described an HPLC method for the determination of malachite green and leucomalachite green. Recoveries were approximately 75% and all results were recovery corrected. For the experiment they used 156 rainbow trout with an approximate body weight range of 200-300 g. The total weight of all fish was 41.2 kg. The temperature was 9.7 ± 0.1°C. Treatment was performed in a tank with 1000 l water to which 200 mg malachite green was added. The proportions of fish to water corresponded to intensive aquaculture conditions for trout. Duration of treatment was 24 hours. Samples for water analysis were taken every hour. Ten fish were sampled immediately after the end of treatment and groups of six were sacrificed at all other sampling times. The last samples were taken on day 143 after treatment. Homogenized tissue samples were frozen and stored at -30°C prior to analysis. Two parallel smaller experiments were carried out in smaller tanks and with smaller fish densities and a treatment concentration of 0.1 mg/l. for methodological studies.

The initial concentration in water of malachite green was 205 mg/m^3 and decreased to 5 mg/m^3 in 24 hours following an exponential term. Thus 97.6% of the malachite green disappeared and was probably taken up by the fish because in one of the smaller experiments 80% of the malachite green that had disappeared from the water bath was found in the fish. About 33% of the malachite green which was taken up was found in muscle. At the end of the treatment period the total concentration of malachite green plus leucomalachite green was 910 ± 243 µg/kg (n=10). The concentration of the parent drug was 86.3 ± 54.4 µg/kg (n=6). On subsequent days the concentrations of the parent drug rapidly decreased and the between fish variability increased.

A graph provided in the original paper shows that the decrease in the concentration of malachite green did not follow a mono-exponential term. However, the group of data points describing the depletion of the leucomalachite green comes closer to a log-linear curve. The concentrations measured in the fatty tissue were very high. Therefore the authors determined the fat content of the muscle samples.

For the muscle samples taken during the first 87 days classified according to fat content they found a very high correlation between fat content and concentration of leucomalachite green in muscle and a decrease of the rate of depletion of leucomalachite green in the groups of fish with the highest fat contents. For fish with the highest fat content the elimination half life of leucomalachite green was 43.3 days. The almost complete uptake of the malachite green shows that the compartment fish was still far from any saturation in this experiment.

Allen (1990) applied colorimetric analysis to samples of muscle, eggs and fry of malachite green treated Atlantic Salmon (*Salmo salar*) and Chinook Salmon (*Oncorhynchus tshawytscha*). Fish had been treated 10 to 47 times with a solution containing 1 mg/kg of malachite green oxalate for one hour. Samples were obtained 1 to 18 days after the last treatment. Residues were extracted with a mixture of 85% ethyl alcohol, 10% formalin and 5% acetic acid. Following extraction in the dark, centrifugation and filtering absorbance at 615 nm was measured in the extracts. The method was not validated. The author states that concentrations of residues in muscle of Atlantic salmon showed no relation with the number of treatments and the concentrations in both species depended only on the elapsed time after the last treatment. Since the methodology is inadequate for the determination of malachite green the numerical results published by the author are most likely of little value.

A paper in Thai language (Amornchai Somletchaoen) for which only an English summary is available reports on the persistence of malachite green in tilapia. Sixty juvenile tilapias of an average body weight of 24.1 ± 6.8 g were exposed to malachite green at two therapeutic doses, 0.1 for 24 h and 0.2 for 1 h at a water temperature varying between 23.5 and 26.0°C. The fish were then transferred into clean water and 3 fish were collected at 0, 6, 12, 24, 72, 120, 168 and 360 h post exposure for the determination of malachite green and leucomalachite green residues in muscle tissues. A LC-MS-MS method was used with LOD of 2µg/kg. Following treatment with the high therapeutic dose, highest average concentrations of malachite green and leucomalachite green were 35.6 ± 5.8 and 32.2 ± 17.5 µg/kg, respectively. Malachite green depleted to 0.4 ± 0.15 µg/kg within 24 hours while leucomalachite green was 1.5 ± 0.7 µg/kg after 120 hours. After treatment at the lower dose the highest average concentration of malachite green and leucomalachite green 4.6 ± 1.8 and 30.6 ± 2.6 µg/kg, respectively. The concentration of malachite green was 2.0 ± 0.35 µg/kg at 72 h and not detectable 168 h after treatment. Concentrations of leucomalachite green remained stable between 12 to 72 h after treatment. At 360 h after exposure, the average concentration was 3.5 ± 2.3 µg/kg.

A study investigating the metabolic profiles and residues of malachite green in trout tissues was carried out for the United States Food and Drug Administration (Law, 1994). The study was conducted in trout kept in tanks under the following conditions: water temperature (10±2°C), pH (6.0-7.0), hardness (5-10 mg/l), and dissolved oxygen (9±2 mg/l). ^{14}C-Labeled malachite green of a radiochemical purity of 98% was used for the treatment. All experiments and analytical work was carried out under decreased intensity room light. Concentrations in the exposure tanks were maintained by a metering apparatus containing a ^{14}C-MG stock solution at 800 mg/l and delivering 10 ml/min of this solution; the concentration of the treatment solution was 2 mg/kg.

Seventy-two randomly selected trout, each weighing about 350g, were divided into 3 groups of 24 fish and put into three 200-l continuous flow exposure tanks containing 2.0 mg/kg ^{14}C-labeled MG (actual concentrations 1.8 ± 0.2 mg/kg, 1.9 ± 0.3 mg/kg and 1.9 ± 0.2 mg/kg, respectively). A water sample (5 ml) was withdrawn from the exposure tanks every 15 min during the ^{14}C-labeled MG exposure period. After a 1-h exposure, the fish were removed to a depuration tank containing flowing, uncontaminated water. At specific time intervals during ^{14}C-labeled MG exposure and depuration two or three trout were removed randomly from each group of fish and sacrificed. The annexes to the study report provided information on concentration of total radioactive residue in tissue homogenates and ratio of malachite green to leucomalachite green concentrations in an organic extract. From these data the concentrations of malachite green and leucomalachite green in the tissues were calculated. The highest concentrations of residues were found in liver and kidney. Significant concentrations of residues were also found in skin.

The data of the study by Law (1994) representing the time period between the end of the treatment and 505 hours post treatment were subjected to statistical analysis using one exponential term on the basis of the natural logarithms of the residue contents for curve fitting. The following parameters given in Table 3 were obtained by linear regression:

Table 3: Parameters of the linear regression analysis of kinetic residue depletion data in trout muscle.

Parameter	MG	LMG
Intercept	- 0.99747	- 0.01994
Slope	- 0.02461	- 0.00352
Coefficient of correlation	- 0.60012	- 0.73361
Residual variance	0.58312	0.52876

The kinetic data representing the concentrations of malachite green and leucomalachite green between the end of the treatment and 505 hours were subjected to statistical analysis using one exponential term on the basis of the natural logarithms of the residue contents. Depletion half lives were 28 hours for malachite green and 197 hours for leucomalachite green. The kinetic parameters including the variance of the data were used to perform estimates of dietary exposure to malachite green (see below). The results obtained for muscle are summarised in Figures 6 and 7. The data are the same in the two figures. In Figure 6 the time axis is given on a logarithmic scale in order to enable better discrimination of the treatment phase and the phase after treatment.

Figure 6: Kinetics of malachite green and leucomalachite green in trout muscle.

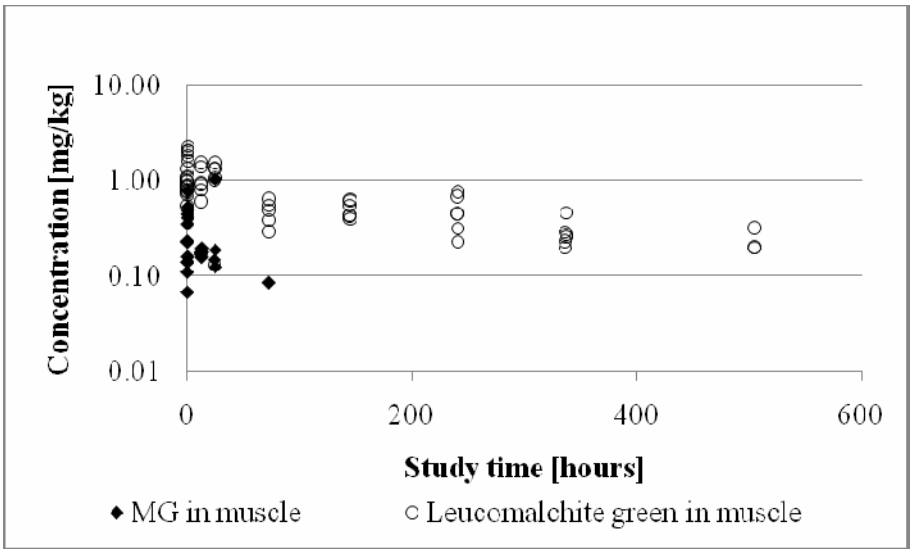
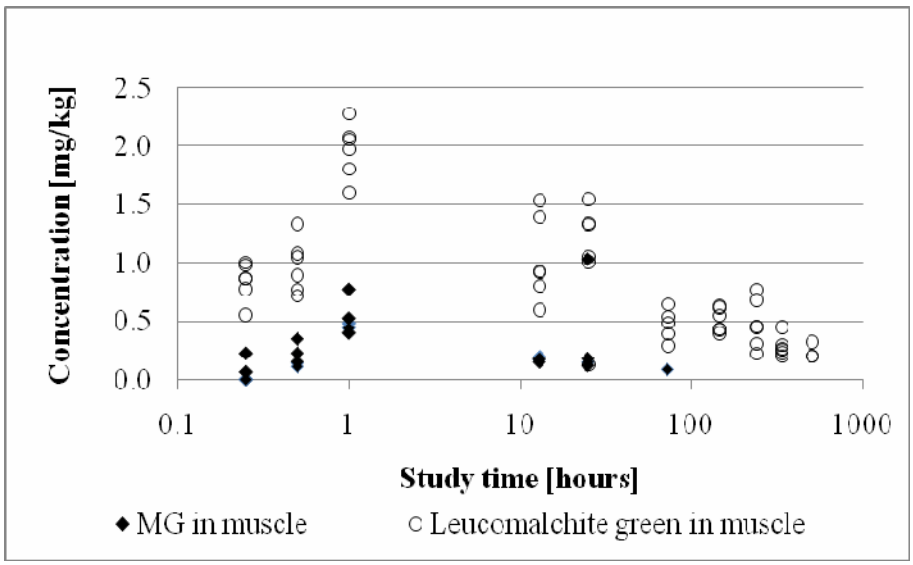


Figure 7: Kinetics in trout muscle – logarithmic time scale.



The following table of results of trials is compiled from the above mentioned publication of Sudova, et al. (2007) that cites the data after Mitrowska and Posyniak (2005).

Table 4: A selection of treatment conditions cited after Mitrowska and Posyniak (2005).

Fish species	Average body weight	Duration of bath [hours]	Water temperature [°C]	pH of bath	Time after treatment [days]	Concentration in muscle tissue [µg/kg]	
						Malachite green	Leucomalachite green
Eel	4.1	24	25	6.9	62	2	139
					80	< LOD	28
					100		15
	0.3				330		< LOD
Channel catfish	600	1	21	7.1	14	12	518
					62	< LOD	19
	580		22	7.2	14	6	310
Rainbow trout	1350		21	7.0	1	73	289
			12	7.8	5	15	230
	0.1	72	9.7		40	1	20
					140	< LOD	
		144	13		300		2

Unsystematic small trials conducted in the context of method development studies

Some information on residue behavior was generated in a less systematic manner in the context of the development of analytical methods. The below section summarized a selection of these studies.

Allen, et al. (1994) carried out recovery experiments in order to assess the performance of a method and treated 6 adult rainbow trout (range of body weights was 1200-1500 g) in well water of pH 7.8 with 1 mg/l of ^{14}C malachite green for 1h . Residues were determined by combustion analysis in fillets (with skin left on) immediately after exposure and after 5 days withdrawal period. Both fortified homogenates and homogenates from treated fish were extracted and analysed after cleanup using HPLC, collection of radioactive fractions and liquid scintillation counting. The results are summarised in Table 5.

Table 5: Results of the study of Allen, et al. (1994).

Material	Days after treatment	Original concentration [mg/kg]		Found in extract		Composition of extract [%]		
		Total residue after combustion	Fortification level with MG	[mg/kg]	%[1]	MG	LMG	Unknown
Fish 1, muscle	0	1.3		0.8	62	29	45	25
Fish 2, muscle				1.1	85	26	49	24
Fish 3, muscle				1.0	77	34	45	21
Fish 4, muscle	5	0.5		0.5	100	3.0	46	51
Fish 5, muscle				0.3	60	3.0	33	64
Fish 6, muscle				0.3	60	1.8	40	59
Egg homogenate			1		85	84	7	9
			0.5		98	81	10	9
Fry homogenate			0.65		68	76	11	13
Muscle homogenate			1		66	11	89	

[1.] The values for the six incurred tissues are calculated from the data; they are not given in the original paper.

The following results obtained with trout muscle are interesting:

- Recoveries from incurred muscle tissues were lower than from fortified homogenates;
- A significant fraction of the radioactive residue was of unknown structure. This fraction increased from approximately 23% immediately after treatment to approximately 58% on day five after treatment.
- Malachite green added to homogenates was largely reduced to leucomalachite green.
- The original solution of malachite green remained unchanged if processed by the same procedure in the absence of tissue homogenate.

Andersen, et al. (2005) developed an analytical method based on liquid chromatography in which leucomalachite green is oxidised prior to cleanup with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). Most of the DDQ is consumed by the sample extract. A high excess is needed; however, excess oxidation and formation of breakdown products has to be avoided. The study included the determination of residues in salmon that had been treated with malachite green. Fish (weight not given) were treated in a water bath (volume not given) with 0.01 mg/l malachite green for one hour (temperature not given). The fish were returned to clean water and sampled 2 and 4 hours after the end of exposure. The concentrations (sum of malachite green and leucomalachite green) were found in Table 6. The authors discuss the lower recoveries of spiked malachite green compared to leucomalachite green as a possible explanation of the higher concentrations found at the later time point. Mean recoveries for malachite green were approximately 71% over a concentration range of 1-10 µg/kg. For the same concentrations of leucomalachite green recoveries averaged 95.4%.

Table 6: Results of the study of Andersen, et al. (2005).

Hours after treatment	Concentration [µg/kg]			
2	29.8	35.2	36.3	35.3
4	48.0	47.6	36.9	52.5

The same group published a paper in which a similar method was used, but results were confirmed with LC-MS (Andersen, et al., 2006). The method was validated for catfish, trout, tilapia, basa, salmon, and shrimp. Incurred tissues were also analysed. For this purpose catfish, tilapia, trout, and salmon were exposed to 0.01 mg/l malachite green in water for 1 h. After transfer to clean water samples were analysed at a different time point for each species 16, 16.25, 16.5, and 24 h, respectively, after the end of the treatment. The results are summarised below:

Table 7: Results of the study of Andersen, et al. (2006).

Fish species	Hours after treatment	LC-VIS		LC-MS	
		mean	sd	mean	sd
		[µg/kg]			
catfish	16	32.2	2.2	31.3	2.7
tilapia	16.25	1.9	0.13	2.1	0.3
trout	16.5	27.1	1.4	28.6	1.1
salmon	24	26.4	0.84	27.4	2

Bergwerf and Scherpenisse (2003) published a method for the determination of malachite green in aquatic animals which is based on HPLC or LC-ESI-MS-MS (for confirmation). The mobile phase was pumped through a pre-column oxidation reactor and effluent was oxidised in a post-column reactor. The authors made the observation that the time interval between spiking of a homogenate and further processing influenced the recovery of malachite green significantly, but had little influence on recoveries of leucomalachite green.

Table 8: Results of the study of Bergwerf and Scherpenisse (2003)

Time [min]	Recovery [%]	
	Malachite green	Leucomalachite green
1	81	100
15	63	96
30	60	93
60	54	93
120	45	95

Malachite green was apparently not reduced, but possibly degraded, since the chromatograms showed satellite peaks next to malachite green. The method was optimised for leucomalachite green. Forty-eight samples of trout, eel and salmon were collected at retail level and on fish markets. Approximately 50% were tested positive for leucomalachite green.

Doerge, et al. (1998b) described LC methods for the simultaneous quantification of malachite green and leucomalachite green using isotope dilution mass spectrometry. In addition they characterised metabolites derived from malachite green and leucomalachite green found in catfish and trout. Mature catfish of approximately 0.5 kg bw were exposed for one hour in a 40 l tank to 1 mg/kg malachite green at 25°C and pH 7.2. The fish were briefly rinsed and transferred to fresh clean water. Fish were killed 24 hours after dosing and fillets (skin removed) were blended and stored at -60°C prior to

analysis. Leucomalachite and malachite concentrations in muscle of treated catfish were 1030 and 590 µg/kg, respectively. Trout were purchased in 1994-1995 from retail outlets in the UK. Blended tissues were spiked with d5-leucomalachite green and $^{13}C_6$-malachite green. Recoveries of the internal standards were about 34 – 70% (n=12) for malachite green and 64-86 % (n=12) for leucomalachite green. The concentrations of incurred residues ranged from 0.4-3.4 µg/kg for malachite green and 9-96 µg/kg for leucomalachite green. Leucomalachite green was present at much higher concentrations (range 12- to 38-fold).

Halme, et al. (2007) proposed an LC-ESI-MS/MS method for confirmation of residues of malachite green and leucomalachite green in trout. D5-leucomalachite green was used as internal standard. They analysed 34 fish monitoring samples of which eight contained malachite green residues. Only the range of the results is given (0.35-1.34 µg/kg of leucomalachite green).

Roybal, et al. (1995) developed a method for the determination of malachite green and leucomalachite green by SPE, HPLC, post-column oxidation and detection at 618 nm. In this context they analysed catfish exposed to 1 mg/kg malachite green oxalate for 1h at 21°C and pH 7.0. The treated and rinsed fish were place into separate aquaria equipped with activated carbon filters. Fish were sacrificed and analysed at 0, 2, 4, 8, and 24 hours after placement in individual aquaria.

Table 9: Results of the study of Roybal, et al. (1995).

Hours after treatment	Concentration [µg/kg]				Replicates
	Malachite green		Leucomalachite green		
	mean	s.d.	mean	s.d.	
0	486	23.4	632	23.6	4
2	190	18.8	703	30.8	4
4	187	23.7	748	30.0	4
8	111	12.8	450	30.7	4
24	73.4	7.5	289	19.8	4

Scherpenisse and Bergwerff (2005) published a method for the determination of residues of malachite green in finfish by LC-MS/MS. Recoveries for malachite green were very low in most fish matrices. Recoveries for leucomalachite green were from 86 to 105%. They used the method to analyse nineteen samples including pangasius, salmon, shrimps and trout bought in local shops. Residues were found in three of the samples (trout 24 and 0.15 µg/kg, pangasius 7 µg/kg).

Turnipseed, et al. (2005) proposed an analytical method in which leucomalachite green is oxidised to malachite green before the SPE extraction step of cleanup and final LC-MS determination. They used the method to analyse two samples of treated salmon (10 µg/l for 1 hour). They found 34.6 µg/kg in a fish 2h after treatment and 44.3 µg/kg in a fish 4 h after treatment.

ESTIMATION OF DAILY INTAKE

In the open literature, well conducted residue studies suitable to predict the concentration–time course of residues of MG in fish are available for only two species, the rainbow trout and the channel catfish. Only for trout were sufficient individual animal data available to perform a statistical evaluation.

Useful information on frequency of occurrence and levels of residues can primarily be obtained from monitoring activities or from well supervised trials conducted under field conditions. The following discussion analyses the selected data for estimation of exposure.

In the UK, approximately 400 trout samples were analyzed in three surveys between July 1993 and March 1995. Sixty-seven samples contained malachite green at concentrations of 2-50 µg/kg. The analytical method did not pick up leucomalachite green. In a survey of retail trout in 1996, malachite

green was detected in 15 of 208 samples. In 1997 there was only one trout sample out of 137 that contained malachite green. A change in methodology was introduced in 1997 and subsequently malachite green and leucomalachite green were measured. When thirty-one randomly taken samples of the 1997 survey of which 29 were negative were re-analyzed with the new method, seven became positive. The new method was applied to the 27 samples taken in 1998. One contained both malachite green and leucomalachite green. In five samples only leucomalachite green was found (COT, 1999). Thus, the introduction of new methodology increased the number of positives. The individual results of the non-compliant samples are given in the cited COT document.

The Veterinary Residues Committee established in 2001 in the UK published the results of all statutory and non-statutory surveillance schemes. For non-compliant samples the individual numerical values found are also given. The presentation of the data was initially such that it was not possible to find out cases in which both residues were found in the same sample. The data are available on the internet. When the results of the 2001-2006 plans were evaluated more than 2300 samples analyzed for malachite green residues have been found. The main fish species covered were trout and salmon, including imports. Occasionally the data are scheduled under "imported farmed fish".

Another useful data set is reported from Denmark. Rasmussen (2007) reported on findings of malachite green in fish in Denmark from 1988 to 2005. 446 plus 95 "targeted samples" were taken. 48 plus 82 "targeted samples" were positive. The author gives individual results for six samples and ranges for the rest of the positives. Unfortunately the individual data were therefore largely not available to the Committee.

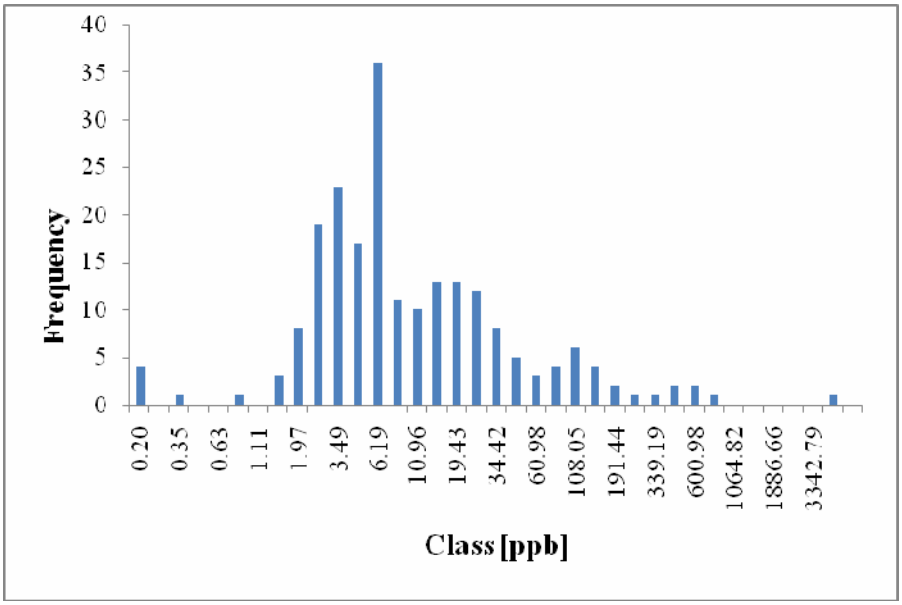
Of 3277 samples selected from these reports, 222 samples were reported positive for malachite green in the range from 0.2 to about 600 µg/kg fish muscle. For many of the samples it cannot be defined what malachite green means (malachite green, leucomalachite green, or the sum of both or just a number because the method was inadequate). Most likely the true concentrations were higher than the results obtained and a significant fraction of the negatives were probably false negatives.

An exhaustive search all public sources of information for data on residues of malachite green is impractical. Many fish species currently moving in international trade and commonly eaten in many parts of the world are not covered. It is not known to what extent results of random sampling and of biased sampling is mixed. It is not known whether any recognised sampling plan has been used in the sampling of lots and which of the individual results given have been obtained from the same lot. The currently published surveillance data are not transparent enough to use it for intake estimates.

The more systematically collected data of the UK from the above described activities can be used to estimate a worst-case figure of upper limit of intake of malachite green resulting from illegal uses. These were monitoring data (spanning from 1995 to 2006) published in the United Kingdom on the occurrence of MG and LMG in fish muscle. If both substances were found in a sample a calculated sum could be determined. The estimated mean level found in the positive samples was 30.7 µg/kg fish muscle and the level at the 97.5th percentile to be 138 µg/kg. Assuming the daily consumption of fish to be 300 g per person, the daily exposure to the sum of malachite green and leucomalachite green can be calculated to be 9.2 and 41 µg/per person at the mean and 97.5th percentile, respectively. For a 60-kg person, this would be equivalent to 0.15 µg/kg bw per day and 0.69 µg/kg bw per day, respectively.

The Figure 8 below shows the frequency distribution of the levels found in the positive samples out of the 3277 samples selected from the above mentioned programs.

Figure 8: Non-representative frequency distribution of illegal residues of malachite green in fish.



The assumption of consumption of 300g of fish contaminated with malachite green and leucomalachite every day for a lifetime was used (a highly conservative assumption). In addition, it was assumed that the concentrations of malachite green and leucomalachite would not change during cooking of the fish.

The study by Law (1994) also was suitable to use for performing a dietary exposure estimate. Such an estimate provides some information on the order of magnitude of likely human exposure to residues of malachite green in case the drug would be authorized for treatments comparable to those performed by the authors.

Depletion half-lives of 28 hours for malachite green and 197 h for leucomalachite green were determined. The kinetic parameters, including the variance of the data, were used to calculate model intakes for every day of 80 years of a human lifespan, assuming daily consumption of 300g of fish muscle. For this purpose 29220 approximately log-normally distributed random numbers were generated for each time point of interest ranging from the predicted value of the regression line minus four times the residual variance to the same predicted value plus four times the residual variance. These calculations were repeated for a number of assumed slaughter times of the fish, ranging from 1h (end of treatment) to 500 h. The results were expressed in mg malachite green/leucomalachite green/kg of human body weight. The minima, maxima and several percentiles, including the median of these estimated daily intakes, were calculated. The median was used for an assessment of chronic intake. The median daily intake of leucomalachite green declined from 7.3 µg/kg bw at hour 1 to 0.87 µg/kg bw at 500 h. Results of an intake assessment for malachite green and leucomalachite green are shown in Table 11 below.

The Committee considered that the assumption of consumption of 300 g of fish contaminated with malachite green and leucomalachite green every day for a lifetime made these estimates highly conservative. In addition, it was assumed that the concentrations of malachite green and leucomalachite green would not change during cooking of the fish. However, that may not be the case.

Mitrowska, et al. (2007) investigated the stability of malachite green and leucomalachite green in muscle of treated carp under various conditions of cooking. The initial concentrations of the residues were approximately 200µg/kg. Leucomalachite green was much more stable than the parent compound. Microwaving was the most effective way to partly destroy the incurred residues. The authors published time curves of the degradation. The end results are summarised in Table 10.

Table 10: Stability of malachite green and leucomalachite green under various conditions of cooking.

Sample	Procedure	Temperature [°C]	Duration [min]	% reduction	
				MG	LMG
Residues in carp muscle	Boiling, baking		15	54	0
	Microwave		1	61	40
Standard solutions	Boiling water	100		0	0
	Cooking oil	150	10	49	
			90	97	
			120		0
		210	10	97	18

Table 11: Results of an intake assessment for malachite green and leucomalachite green.

	Part I – Estimated intake at various theoretical slaughter times of fish														
	1h	**1.6h**	**2.4h**	**3.8h**	**5.9h**	**9.2h**	**14.3h**	**22.4h**	**34.9h**	**54.3h**	**84.7h**	**132.0h**	**205.8h**	**320.8h**	**500.0h**
	Intake of Malachite green [µg/kg bw per day]														
min	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0
P50	1.8	1.8	1.7	1.7	1.6	1.5	1.3	1.1	0.8	0.5	0.2	0.1	0.0	0.0	0.0
P90	3.8	3.8	3.7	3.6	3.4	3.1	2.8	2.3	1.6	1.0	0.5	0.2	0.0	0.0	0.0
P95	4.7	4.7	4.5	4.4	4.2	3.8	3.4	2.8	2.0	1.3	0.6	0.2	0.0	0.0	0.0
P97.5	5.6	5.6	5.4	5.2	5.0	4.6	4.0	3.3	2.4	1.5	0.7	0.2	0.0	0.0	0.0
P99	6.9	6.9	6.6	6.5	6.1	5.7	5.0	4.1	2.9	1.8	0.9	0.3	0.0	0.0	0.0
max	15.6	16.2	21.1	15.2	14.2	14.1	11.8	9.8	10.4	3.8	2.0	0.6	0.1	0.0	0.0
	Estimated intake of Leucomalachite green [µg/kg bw per day]														
min	1.5	1.4	1.5	1.3	1.5	1.1	1.3	1.2	0.9	0.8	0.8	0.5	0.3	0.3	0.1
P50	7.3	7.3	7.2	7.1	6.9	6.7	6.4	6.0	5.5	4.8	4.0	3.3	2.5	1.7	0.9
P90	12.6	12.6	12.3	12.2	12.0	11.7	11.3	10.7	10.0	8.9	7.8	6.4	4.8	3.3	1.7
P95	14.7	14.7	14.5	14.6	14.1	13.8	13.3	12.6	11.7	10.7	9.3	7.7	5.8	4.0	2.1
P97.5	16.9	17.0	16.6	16.8	16.4	15.8	15.3	14.5	13.5	12.5	11.0	9.1	6.9	4.6	2.4
P99	19.7	19.5	19.6	19.7	19.1	18.9	18.3	17.3	16.0	15.0	13.4	10.9	8.3	5.6	3.0
max	48.5	42.4	36.0	39.7	36.2	34.6	38.0	38.9	31.5	32.0	30.3	22.6	16.6	14.3	6.4
	Estimated intake of the sum of Malachite green and leucomalachite green [µg/kg bw per day]														
min	1.7	1.6	1.7	1.4	1.7	1.3	1.4	1.3	1.0	0.8	0.8	0.5	0.3	0.3	0.1
P50	9.1	9.1	8.9	8.7	8.5	8.2	7.7	7.1	6.3	5.3	4.3	3.4	2.5	1.7	0.9
P90	16.4	16.4	16.0	15.8	15.4	14.8	14.1	12.9	11.6	9.9	8.3	6.6	4.9	3.3	1.7
P95	19.4	19.4	19.0	19.0	18.3	17.6	16.7	15.4	13.8	12.0	9.9	7.9	5.9	4.0	2.1
P97.5	22.5	22.5	22.0	22.0	21.4	20.4	19.3	17.8	15.9	14.0	11.7	9.3	7.0	4.6	2.4
P99	26.7	26.4	26.2	26.3	25.2	24.6	23.3	21.4	18.9	16.8	14.3	11.2	8.4	5.6	3.0
max	64.1	58.6	57.1	54.9	50.4	48.8	49.8	48.7	42.0	35.8	32.3	23.2	16.7	14.3	6.4

Note: For ease of reading and formatting the data, the table entries are rounded values using standard rounding techniques.

Results obtained from other surveys

Only a few examples of the type of information available from surveys can be given here in order to facilitate the discussion of the limited usefulness of such results in the context of intake assessments.

Example 1*:* The Centre for Food Safety of the government of the Hong Kong special administrative region frequently informs consumers about findings of noncompliant foods. In four separate reports from December 2006 to November 2007, 29 positives were reported from 130 samples of varying sea and fresh water samples collected at import and local markets at concentrations of 14 – 480 µg/kg.

Example 2: Reports on monitoring malachite green in aquatic species are available from Australia and New Zealand (FSANZ, 2005). The 60 samples of 7 species of fish were from eight countries of origin. The LOQ for malachite green was 2µg/kg using an LC-MS/MS method. The range of malachite green concentrations was from 4 – 138 µg/kg.

Example 3: The Canadian total diet study (1993-2004) collected shrimp and fish samples of various species from various sources to prepare 30 composite samples for analysis of residues of veterinary drugs (Tittlemier, 2007). Fish were baked at 230 °C for approximately 10 minutes. Shrimp were boiled in tap water. It is unlikely that malachite residues are stable under these conditions of sample preparation. It would have been useful to include composite samples of raw fish. The composite samples were frozen and stored at -20 °C until analysis in 2005. The report makes no statement on the stability of residues over such long storage times at relatively high temperatures. The LC-MS/MS methods used for the determination of malachite green and leucomalachite green had a limit of detection of 0.15 µg/kg. Leucomalachite green was found in three of the compos*ite* samples (freshwater fish 2002 and 2003, 0.95 and 0.73 µg/kg; shrimp 2002, 1.2 µg/kg).

Results obtained from residue depletion studies

Residue depletion studies are only available for the rainbow trout, the channel catfish and tilapia. Only one of all the residue studies discussed above is suitable to predict the time course of residues of malachite green in fish. Table 12 summarises some selected characteristics of four major kinetic and residue studies. Despite the large differences in bath size the ratio of fish weight to bath weight is similar; however, there are large differences in the amounts of drug available per fish. This is most likely a critical factor in studies with prolonged exposure times. The figure is lowest for the study with the longest exposure time.

Only one study, conducted by Law (1994), replaced the malachite green taken up from the bath by the fish. The main argument against using the data of the Alderman and Clifton-Hadley study (1993) is that the otherwise well designed study exhibited methodological deficits and used an analytical method that was not valid for the purpose. The Plakas, et al. (1996) study is excellently designed and conducted, but the individual fish residue data were not available. The Bauer, et al. study could have been well used for an observation period similar to the exposure time in the other study; however, individual data were also not available. Overall, only limited conclusions are possible for this study because most of the malachite green was used up by the fish during exposure to the bath.

Table 13 highlights some possible impact on results of bath treatment studies regarding the study designs. For the calculations in the table the physiological data for trout discussed in a previous section were used. Three options regarding ventilation volume and breaths/min were calculated for the Alderman and Clifton-Hadley study (1993), one alternative is given for the Bauer, et al. study (1988). From the data presented the design of the Alderman and Clifton-Hadley study was largely acceptable, however, it was not the case for the Bauer et al. study. This is further substantiated in Figure 10.

Table 12: Selected characteristics of four kinetic residue studies.

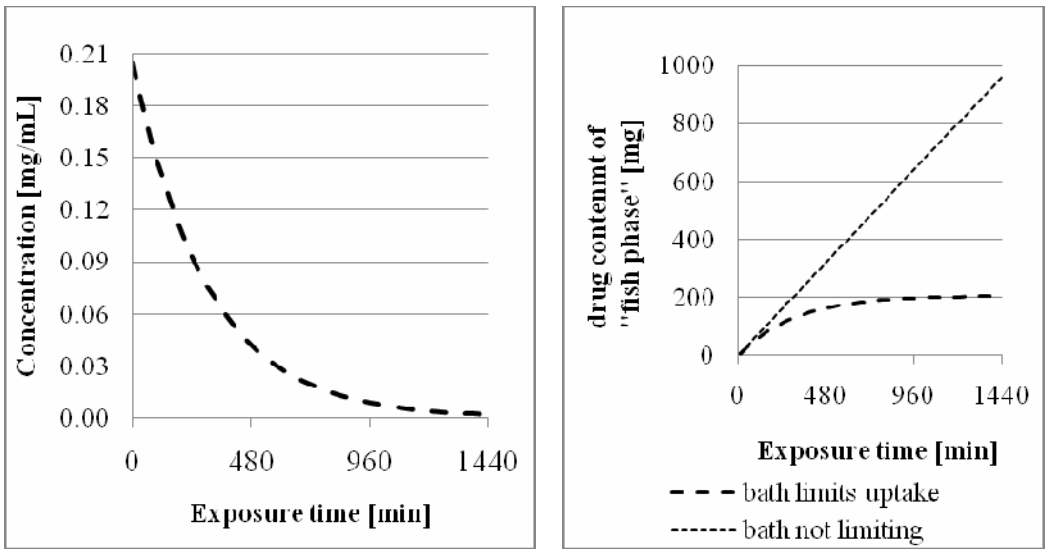
Authors and year	Species	Volume of the tank used for treatment [L]	Initial concentration of malachite green [mg/L]	Average temperature [° C]	Duration of exposure [min]	Estimated maximum weight of all fish in the tank	Ratio bath weight to fish weight	Initial amount of drug available [of fish]
Bauer, et al., 1988	Rainbow trout	1000	0.21	9.7	1440	41.2	24.27	4.98
Alderman and Clifton-Hadley, 1993	Rainbow trout	725	1.6	16 8	40	28.92	25.07	40.11
Plakas, et al., 1996	Channel catfish	100	0.8	21	60	3.5	28.57	22.86
Law, 1994	Rainbow trout	200	2.0		60	8.4	23.8	47.6

Table 13: Fish-physiological aspects of selected kinetic residue studies.

Ventilation volume mL/min	Stroke volume [mL]	Breaths/min	Initial MG concentration [mg/L]	Tank volume [L]	Total amount of MG in tank [mg]	Exposure time [min]	Total number of breaths per animal	Number of fish	Stroke volume of all fish together [mL]	Total inspired water during exposure [L]
22	0.5	44	1.6	725	1160	40	1760	120	60	105.6
37	0.5	74	1.6	725	1160	40	2960	120	60	177.6
49	0.5	98	1.6	725	1160	40	3920	120	60	235.2
22	0.5	44	0.205	1000	205	1440	63360	150	75	4752

For a primitive modelling exercise it was assumed that the inspired water is completely cleared from malachite green which means the amounts inspired with a stroke remain in the fish. On this basis the following two graphs (Figure 10) were prepared modelling the situation of the Bauer, et al. study. The data were generated by dissecting the whole uptake process into the number of elementary steps dictated by the above given total number of breaths per animal.

Figure 10: Modelling of some aspects of the Bauer et al. (1988) study.



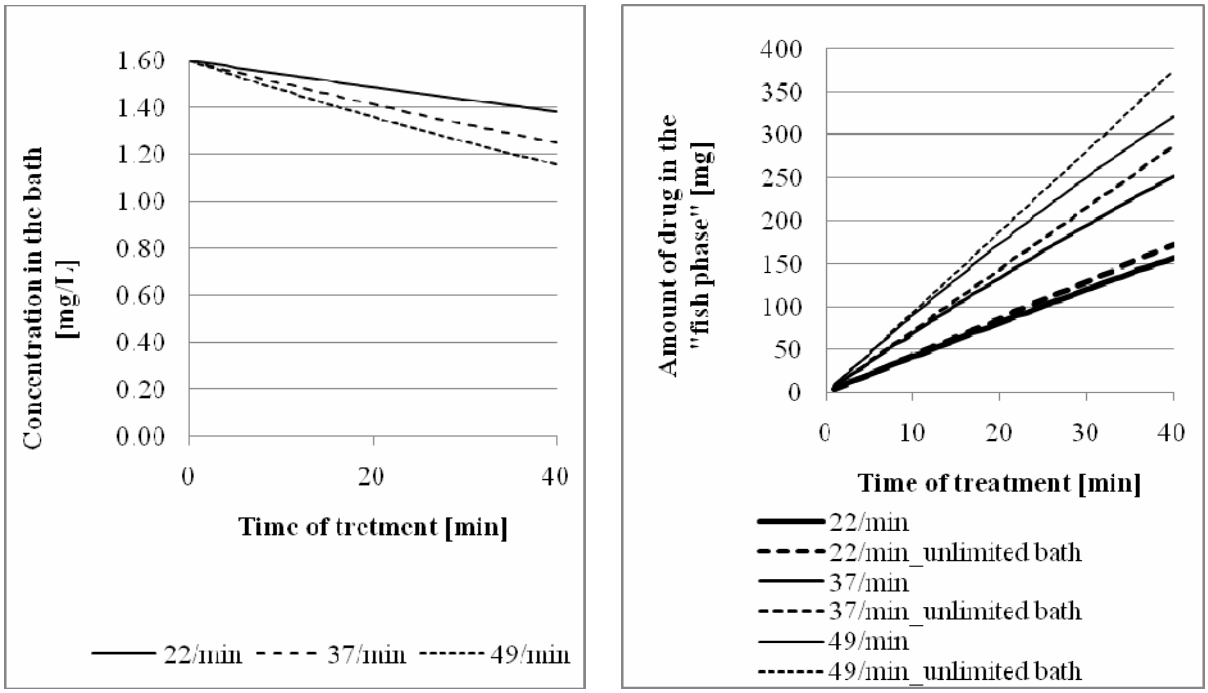
Left side: changes in the drug concentration in the bath; right side: uptake during exposure in a limited bath volume and limited amounts of available malachite green.

The modelling on the left side of Figure 10 predicts that the bath volume is insufficient for an exposure experiment of 1440 minutes duration involving 150 fish. The right modelling experiment predicts that the uptake of malachite green by the fish will be limited by the amount of available drug and therefore, could lead to the wrong interpretation that the uptake capacities of the fish were saturated. The model predicts a final concentration of 2 mg/m^3; the authors reported 5 mg/m^3. Also the predicted amounts of residues in the fish are in the same order of magnitude as experimentally determined by the authors. Although the study has provided some remarkable results it is not representative for the treatment of rainbow trout at 0.8 mg/l in a bath.

Similar calculations were performed using the information from the Alderman and Clifton-Hadley study (1993) and are summarized in Figure 11 below. The graphs show that the concentration in the water bath decreased by approximately 25% in the worst case model using the highest ventilation volume. If one compares the predicted amounts taken up by the fish it appears that the scenario with the lower ventilation volume fits better to the order of magnitude of initial tissue residue concentrations given by the authors. Thus the study was well designed but suffered from a number of weaknesses discussed in a previous section.

It was not possible to perform similar modelling with the information provided in the Plakas et al. study (1996). However, this was also not necessary because the authors were prudent to use a fresh bath for every set of five fish treated and they report that the concentration of malachite green in the bath decreased by only approximately 15% during treatment.

Figure 11: Modelling of some aspects of the Alderman and Clifton-Hadley study (1993).



Left side: Changes in bath concentration as function of strokes/min; Right side: Changes in drug uptake as function of strokes/min.

METHODS OF ANALYSIS FOR RESIDUES IN TISSUES

Sample preparation

Animal tissues

Typically, residues of malachite green and leucomalachite green are extracted from (2 - 5 g) homogenized animal (catfish, eel, rainbow trout, salmon, tropical prawn, turbot, carp, tilapia, tiger shrimp) tissues (raw or cooked muscle samples). Amber flasks are generally used in the protocol to avoid photo-degradation phenomena that would occur during sample preparation.

The typical protocols for malachite green and leucomalachite green extraction involve vortex mixing or shaking in acetonitrile mixtures (extraction is generally performed over 3 to 15 minutes from 500 to 4000 rpm); the inclusion of anti-reductants and radical scavengers has been common practices. Several acetonitrile buffer extractions protocols are reported: mixture of McIlvaine buffer (pH 3.0, 18.9 ml 0.2 *M* sodium hydrogen phosphate and 81.1 ml 0.1 *M* citric acid) and 12 ml acetonitrile (Bergwerff and Scherpenisse, 2003; Dowling, et al., 2007); acidic (0.1% acetic acid) acetonitrile with NaCl (Hernando, et al., 2006); 0.1M ammonium acetate pH 4.5 and acetonitrile (Andersen, et al., 2005, 2006; Tarbin et al., 2008; Hall, et al., 2008). Other mixtures such as hydroxylamine solution (25%), 0.5 ml of *p*-toluenesulfonic acid solution (1 M) and 5ml of acetate buffer (0.05 M, pH 4.5) are also reported (Mitrowska, et al., 2005; Mitrowska, et al., 2007; Andersen, et al., 2008).

Purification is generally performed with SPE and /or liquid/liquid extraction with dichloromethane. Clean up over SPE may be carried out over aromatic sulfonic acid solid-phase extraction columns (Bergwerff and Scherpenisse, 2003; Anderssen, et al., 2008). Malachite green and leucomalachite green are eluted with the following mixture: 2.5 ml 1.0 mg/ml methanolic ascorbic acid, 20 ml 50 mM sodium perchlorate containing 25 mM sodium acetate and 25 mM 1-pentanesulfonic acid adjusted to

pH 4.0 with acetic acid, and 27.5 ml acetonitrile (Bergwerff and Scherpenisse, 2003). Other elution conditions have been reported on the same SPE cartridges: 90% (v/v) methanol, 5% (v/v) of 1mg/ml ascorbic acid and 5% (v/v) of 25% (m/v) aqueous NH_4OH (Scherpenisse and Bergwerff, 2005). Purification is also reported on Strata SCX (strong cation-exchange) disposable columns with a mixture containing acetonitrile and ammonium hydroxide (25%) (90/10) (Mitrowska, et al., 2005; Tarbin, et al., 2008) or with citrate buffer/acetonitrile (Stubbings, et al., 2005).

Few papers report the development of Molecularly Imprinted Polymers (MIP) based SPE for selective purification of malachite green from fish water and fish feed samples. Malachite green is used as template, methacrylic acid (MAA) as the functional monomer and ethylene glycol dimethacrylate (EGDMA) as the linking agent (Li, et al., 2008). Eighty percent cross reactivity with leucomalachite green was observed. The use of malachite green as a template might however lead to a bleeding phenomenon.

Solid-Liquid (SLE) extraction methods are also reported for the purification step using Bondesil-NH2, 40 µm particle size (Hernando, et al., 2006).

The literature also reports some protocols with an "*in situ*" quantitative oxidation of leucomalachite green into MG by reaction with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone after the acetonitrile extraction step. Resulting total malachite green is then subsequently purified by solid phase extraction with alumina and propylsulfonic acid phases (Anderssen, et al., 2005, 2006).

HPLC analysis - Screening tests

Current methods for the determination of malachite green and leucomalachite green in fish tissues or water are based on liquid chromatography (LC), mainly with visible (VIS)/fluorescence (FLD) on-line detections. The parent compound has λ_{max} at 620 nm, whereas the leuco form has λ_{max} at 265 nm, making it difficult to determine malachite green and the leuco form using the same conditions. In practice, the absorbance detector is set at 620 nm (Bergwerff and Scherpenisse, 2003; Mitrowska, et al., 2005, 2007, 2008; Anderssen, et al., 2008) or 618 nm (Anderssen, et al., 2005; Stubbings, et al., 2005) for malachite green detection while the fluorescence detector is set at λ_{ex} = 265 nm and λ_{em} = 360 nm for leucomalachite green detection (Mitrowska, et al., 2005, 2007, 2008; Anderssen, et al., 2008).

Chromatographic separation is reported on phenyl-hexyl analytical columns fitted with corresponding guard columns; the mobile phase consisting in acetonitrile and acetate buffer (0.05 M, pH 4.5) (70:30, v/v) in isocratic conditions (Mitrowska, et al., 2005, 2007, 2008). The use of reversed-phase analytical chromatographic columns is also reported in this context - ODS-2 (Bergwerff and Scherpenisse, 2003), Alltima C18 (Anderssen, et al., 2005, 2008) with acetonitrile based mobile phases such as mixture of sodium perchlorate containing pentanesulfonic acid and acetonitrile in a ratio 2:3 (v/v) (Bergwerff and Scherpenisse, 2003) or ammonium acetate buffer/acetonitrile 50/50 (v/v) (Anderssen, et al., 2005, 2008, Stubbings, et al., 2008).

Simultaneous LC-VIS determination of both forms is possible by post-column oxidation of leucomalachite green to malachite green to convert the colorless leuco form into the chromophore using cartridge containing lead(IV) oxide (PbO_2) (Allen and Meinertz, 1991; Allen, et al., 1992; Swarbrick, et al., 1997; Rushing. et al., 1995; Tarbin, et al., 1998; Bergwerff and Scherpenisse, 2003). Post column oxidation protocols are also reported (Valle, et al., 2005). Electrochemical oxidation has been used as an alternative to PbO_2 (Rushing, et al., 1997). The determination of both compounds together constitutes a good screening method to confirm the presence of this kind of residue, taking into account that the combined signals will provide a gain of sensitivity. Detection limits reported for LC-VIS measurements are around 1 µg/kg.

More recently, screening tests involving mass spectrometric detection have been reported for the simultaneous measurement of malachite green and leucomalachite green. The sum of malachite green

and leucomalachite green is determined by liquid chromatography coupled to atmospheric pressure chemical ionisation mass spectrometry (LC-APCI-MS) after post column oxidation (Valle, et al., 2005). Detection limit obtained on spiked salmon samples based on ion at m/z 313 is 0.15 µg/kg. Typical recoveries are in the range 70-85%.

Screening tests involving Surface-Enhanced Raman microfluidic sensors have also been reported for water analysis. This kind of biosensor allows fast and sensitive trace analysis of malachite green (Lee, et al., 2007; Lucotti, et al., 2007). Malachite green molecules are adsorbed onto silver nanoparticles while flowing along the polydimethylsulfoxane (PDMS) channel. A quantitative analysis of malachite green is performed based on the measured peak height at 1615 cm^{-1} in its SERS spectrum. Corresponding limit of detection was found around 1-2 µg/kg.

Finally, ELISA tests have also been developed for selective detection of malachite green and the related triphenylmethane dyes in fish and fishpond water (LOD = 0.05 µg/L in water) (Yang, et al., 2007). Performance characteristics are noted below.

LOQ: 0.49 µg/kg (malachite green, UV-VIS) (Andersen, et al., 2008)
LOD: 0.15 µg/kg (MS detection ion m/z 313 (MG+LMG)), 0.15 µg/kg (MG, UV-VIS, (Andersen, et al., 2008); 1 µg/kg (MG + LMG, UV-VIS) (Bergwerff and Scherpenisse, 2003, Andersen, et al., 2006); 1-2 µg/kg (SERS) (Lee, et al., 2007).
CCα: 0.15 µg/kg (MG UV-VIS), 0.13 µg/kg (LMG, FLD) (Mitrowska, et al., 2006)
CCβ: 0.37 µg/kg (MG UV-VIS), 0.32 µg/kg (LMG, FLD) (Mitrowska, et al., 2006)
Linearity: R^2 =>0.995
Precision: RSD 7.7-10.9% (MG, UV-VIS) 7.7-8.4%(LMG, FLD) (Mitrowska, et al., 2006)
Accuracy: 60-64% (MG, UV-VIS), 89-92% (LMG, FLD) (Mitrowska, et al., 2006)
Recovery: 85-90% LMG (Andersen, et al., 2008; Mitrowska, et al. 2005), 60-70% MG (Andersen, et al., 2008, Mitrowska, et al. 2005)

Confirmatory methods

For confirmatory purposes analytical procedures utilize detection by mass spectrometry (MS) with liquid or gas chromatography, which does not demand post-column oxidation of leucomalachite green (Turnipseed, et al., 1995a). However, either the PbO_2 reactor or the "*in situ*" oxidations are used with MS, because detection of malachite green is more sensitive comparing it with leucomalachite green (Tarbin, et al., 1998; Bergwerff and Scherpenisse, 2003).

Gas chromatography coupled to mass spectrometry (GC-MS) analysis

GC-MS analyses were first developed in the mid 1990's to provide confirmatory methods for leucomalachite green in fish tissues (Turnipseed, et al., 1995b). Selected ion monitoring was performed based on five diagnostic ions (m/z 330, 329, 253, 210 and 165).

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis

Chromatographic separation is generally performed on phenyl phases using either a gradient of acidic acetonitrile (0.1% FA)/ water or an isocratic mixture of acetonitrile/acetate buffer (70/30, v/v) as mobile phases (Andersen, et al., 2006, 2008, Mitrowska, et al., 2008). C_{18} phases with 50mM ammonium acetate/acetonitrile or acidic water/acetonitrile as eluents have also been reported (Bergwerff and Scherpenisse, 2003; Scherpenisse and Bergwerff, 2005; Hernando, et al., 2006, Tarbin, et al., 2008).

Atmospheric pressure chemical ionization coupled to ion trap (APCI-IT)

Atmospheric pressure chemical ionization coupled to ion trap has shown to be a very sensitive and selective technique for the analysis of malachite green which is recovered under $[M]^+$ charged species

with a molecular ion at m/z 329 (Valle, et al., 2005). The use of ion trap as mass analyzer is reported since it allows for full collection of product scan data, further increasing the analytical selectivity of the method (Doerge, et al., 1998b). (MS acquisition program = MS^2 scan of m/z 329, width 2 amu, relative. collision energy 48-50%, activation Q = 0.25, activation time = 30 ms, mass range 150-350) (Andersen, et al., 2006, 2008). The product ions include m/z 314 (M^+-CH_3), m/z 313 (M^+-H-CH_3), m/z 285 (M^+-NC_2H_6), m/z 251 (M^+-C_2H_6), m/z 237 (M^+-C_6H_5-CH_3) and m/z 208 (M^+-C_6H_5 NC_2H_6). High collision energy is needed to obtain significant abundance of these ions.

LC-ESI+-QqQ

Analysis with liquid chromatography coupled to an electrospray ionization-triple quadrupole mass spectrometer allows monitoring malachite green as $[M]^+$ with the following transitions: 329.3>165.0, 329.3>208.0, 329.3>313.3 with associated collision energy ranging from 45 to 75 V; leucomalachite green ($[M+H]^+$ is monitored using the following transitions: 331>239 and 331>316 (Bergwerff and Scherpenisse, 2003, Dowling, et al., 2007; Scherpenisse and Bergwerff, 2005; Mitrowska, et al., 2008; Tarbin, et al., 2008).

LC-ESI+-TOF

Applications have also been reported with liquid chromatography coupled to time-of-flight mass spectrometry with electrospray ionization (R = 9500 FWHM) for improved selectivity, especially with regard to matrix effect (see figure 15) (Hernando et al., 2006). However, sensitivity performances of the corresponding method are not compliant with the legislation since the minimum residue performance level (MRPL) is exceeded.

LC-ESI+-LTQ

One application is reported with a linear ion trap as mass analyzer exhibiting similar performances as observed with ion trap or triple quadrupole technologies (Wu, et al., 2007).

Isotopic internal standards (d_5-MG and $^{13}C_6$-LMG) are available and have been used to overcome problems such as matrix suppression during electrospray ionisation (Hall, et al., 2008)

Performance characteristics of confirmatory methods

LOQ: 0.75 µg/kg (Andersen, et al., 2008)[6 ppb (MG), 3 µg/kg (LMG), TOF (Hernando, et al., 2006)]
LOD: 0.2-0.25 µg/kg (Bergwerff and Scherpenisse, 2003, Andersen, et al., 2006, 2008) [2 µg/kg (MG), 1 µg/kg (LMG), TOF (Hernando, et al., 2006)]
CCα: 0.07-0.14 µg/kg (MG), 0.05-0.17 µg/kg (LMG) (Scherpenisse and Bergwerff, 2005; Dowling, 2007) [8 µg/kg (MG), 38 µg/kg (LMG) TOF (Hernando, et al., 2006)][1.2 µg/kg (MG) multiresidue (Tarbin, et al., 2008)]
CCβ: 0.15-0.23 µg/kg (MG),0.08-0.21 µg/kg (LMG) (Scherpenisse, 2005) [13 µg/kg (MG), 65 µg/kg (LMG), TOF (Hernando, et al., 2006)] [2.0 µg/kg (MG) multiresidue (Tarbin, et al., 2008)]
Linearity: $R^2 > 0.995$ in the range 0.5 – 10 µg/kg (Andersen, et al., 2008)
Accuracy: RSD 10%
Recovery: 85-100%

Extraction and Quantification in incurred samples

Although the ability to detect malachite green and leucomalachite green at regulated levels has been dramatically improved by the use of LC-MS/MS and SPE clean-up procedures, the analysis of malachite green in fish tissues remains a challenge, essentially due to issues surrounding extraction and analyte stability. This issue has been reported recently in literature with a high accuracy method

for quantification of malachite green and leucomalachite green in salmon using exact matching isotope dilution mass spectrometry associated to longer extraction time (16h) (Hall, et al., 2008). Results showed that whilst the total extraction and equilibrium of leucomalachite green was achieved in less than 1h, further malachite green could still be extracted up to 16h. This highlights the difference in chemical behaviour of the two analytes in fish matrix and the necessity for longer extraction time. Further work could concentrate on improving the rate of release of malachite green from fish tissue (e.g., enzymatic digestion). In particular the binding of malachite green to proteins might be an issue in extraction efficiency.

Stability of the analytes in incurred samples

Degradation is reported as less than 10% after 12 months storage at -20°C, however, dramatic degradation is observed for malachite green at room temperature (recoveries from 80 to 40 % in 2 hours), and little effects are observed on leucomalachite green. Malachite green and leucomalachite green recoveries are strongly affected by freeze-thawing cycles and storages at +4°C and -20°C.

Degradation products

The metabolite leucomalachite green is not the endpoint of malachite green transformation and the MRPL fixed at 2 µg/kg and the corresponding sum MG+LMG is an underestimate of the actual presence of malachite green residues. Indeed, several studies have shown that malachite green and leucomalachite green are de-methylated by systematic sequential oxidations (Culp, et al., 1999). (Bergwerff and Scherpenisse, 2008) provide a tabular summary of the structures of residues of malachite green identified in treated rainbow trout.

The degradations products may be formed in living fish organisms during enzymatic action but also during photo-oxidative degradation in water (Mitrowska, et al., 2008; Bergwerff and Scherpenisse, 2008). Some identified degradation products in incurred rainbow trout or in water are shown in figure 16 [m/z 315 (N-demethyl-MG), m/z 301 (N,N-didemethyl-MG); m/z 317 (N-demethyl-LMG); m/z 303 (N,N-didemethyl-LMG)] (Mitrowska, et al., 2008; Bergrwerff and Scherpenisse, 2008). In rainbow trout, these derivatives were found to represent about 20% of the total concentration of malachite green residues. Since these demethyl derivatives are also expected, like malachite green and leucomalachite green, to react with DNA, being thus potential carcinogens, the MRPL (2 µg/kg for MG + LMG) (European Commission Decision 2004/25/EC) may therefore be subjected to future revision.

Recent literature indicates that malachite green undergoes three main photolytic degradations under natural sunlight irradiation: N-demethylation, hydroxylation and cleavage of the conjugated structure forming benzophenone derivatives (Perez-Estrada, et al., 2008). More than 20 transformation products have thus been identified. These processes involve hydroxyl radical attack on the phenyl ring, the N,N-dimethylamine group and the central carbon atom. The *Vibrio fischeri* acute toxicity test showed that the solution remains toxic after malachite green has completely disappeared. This toxicity could be assigned, at least in part, to the formation of 4-(dimethylamine)benzophenone (D20), which is considered 'very toxic to aquatic organisms' by current EU legislation.

Degradation of malachite green and leucomalachite green reported and explained by photo-oxidative demethylation, might be prevented or at least reduced during sample preparation by addition of ascorbic acid (Mitrowska, et al., 2005, 2008) or N,N,N',N'-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD) to the analytical matrix (Bergwerff and Scherpenisse, 2008).

Multi-residue method sensitivity

As multi-residue protocols are developed and more reported as a trend in scientific literature (Tarbin, et al., 2008), performances ($CC\alpha$ = 1.2 µg/kg and $CC\beta$ = 2 µg/kg for malachite green in fish tissues) they may not be compliant, in particular with the MRPL (2 µg/kg MG + LMG).

Influence of processing

Effects of various cooking methods (boiling, baking, microwaving) on malachite green and leucomalachite green have been investigated in incurred carp muscles as noted previously (Mitrowska, et al., 2007). A decrease in concentration was observed for malachite green: - 54% after 15 min boiling or baking; leucomalachite green was stable under these conditions. Microwaving induced a loss of both malachite green and leucomalachite green after 1 min (- 61% and - 40%, resp.). Malachite green also appeared to be degraded in cooking oil at 150 °C (50 % in 10 min).

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