Title: USE OF ONE OR MORE CHEMICAL COMPOUND FOR MARKING OF FISH

Abstract: Composition for marking of fish, comprising one or more compounds binding durably to calcium rich structures such as fish-scales, bones, fin rays, teeth and otoliths. The compound or compounds are not poisonous, do not substantially affect the metabolism of the fish, and do not exist naturally in the fish’s environment. At least one of the compounds is easy to detect by, for example, colour or fluorescence. Method for marking of fish by using a composition according to the present invention.
Use of one or more chemical compounds for marking of fish.

The present invention relates to the use of a combination of one or more chemical compounds for commercial, traceable marking of fish, and a composition and a method for such marking, according to the preamble of patent claim 1, 6 and 11, respectively.

Background

In the marine fish farming industry one has until now, not marked every single individual fish commercially, but desires and needs for documentation of the environment towards consumers and different members of the production chain, have created a need for the introduction of an identification system which makes it possible to identify individual fish in the said company, sea plant or net cage, topical. The environmental administration has expressed a desire to mark fish in order to separate wild fish from farmed fish, and to be able to take action in order to limit the escape of farmed fish. The same considerations apply as a result of American and European governments now wishing to introduce marking of farmed fish, on a net cage level. The future demands/needs when considering the environment (spread of disease, escape etc.), will be conclusive for which demands to identification, that will be approved. Identification will in many respects mean that one can trace an individual fish back to the producer company, the age group, area, location and/or net cage the individual fish has been in.

Today, several different commercial marking methods are available on the market. Most of these are based upon internal and/or external physical marking, and individual handling upon marking. Among these are CWT nose-marking, PIT tag, VIT mark, Carlin mark and Floy mark. However, the methods have been developed mainly for identification of a limited number of individuals, and primarily adapted to research needs.

It is also known to use chemical marking by bath treatment, using the marking compound alizarin complexone, and identification by visual detection of "bar code" in the otolith, effected and used in connection with testing and research (Iglesias Rodriguez-ojea 1997; Moen 1996, 2000). A condition for identification of the mark is that the fish is killed, and that the otoliths are taken out for reading.

There is however, still a need for quick and effective marking of a larger or smaller number of individuals in a group of fish. It is also desirable to provide the possibility of, several times, identifying the same individual in a group of fish, by repeated sampling in the production chain or later. In most cases this will demand two types of detection, both a quick gross detection in the field (separate all marked fish from unmarked fish) and a high grade detection (to decide the
marking code of an individual). At present there are no marking methods providing these possibilities and enabling marking in commercial production of fish. To separate the marks from each other, there is a need to produce and detect a large number of stable marking codes. Known methods for chemical marking, have evident limits, in this connection.

Object

The object of the present invention is to provide a composition and a method for marking of fish. The mark to be achieved should be readily available in such a way that it is easy to decide whether the fish is marked, it should also follow the fish from marking to consumption, and if the fish is marked, it should be possible to decide where it came from, without having to kill the fish. Further, a large number of codes should be obtainable, and the codes should be detectable both qualitatively and quantitatively, without having to kill the fish. Moreover it is an object that the method should not be work-demanding, interrupt the fish as an organism or make it unsuitable as food.

The invention

The objects of the invention are achieved by use, composition and method according to the characterizing part of patent claims 1, 6 and 11, respectively. Further advantageous features are given in the respective dependent claims.

The invention concerns use of one or more chemical compounds for marking of fish, and a composition and a method for such marking. With the invention one achieves a mark in the fish, and by means of this, the history of the fish can be decided, and both the producer of the smolt and the fish to be slaughtered, can be stated. Further, it may be possible to state both which fish farming unit the fish was produced by, and which net cage it has been in. A postulation for this is, of course, that there exists a summary of which groups of fish have been marked and in which way. The mark is achieved by utilizing the compound(s) as a mark in structures having low metabolic activity, and that is not in equilibrium with the surrounding weave. Chemical compounds which are naturally present and bound to outer calcium rich structures in such a way that they can be detected and/or analysed later, are considered to be the most suitable.

The term "calcium rich structures" means herein calcium structures in general, and scales, bones, fin rays, teeth and otoliths of fish especially.

Alizarine, calcein and similar compounds are examples of suitable substances for use in chemical marking of fish according to the present invention. According to SNT (Norwegian Food Control Authority) there is no health risk by eating fish marked with alizarin and/or
calcein. Alizarin is a well-documented dye, and exists naturally in the root of the plant *Rubia tinctorum* L. Alizarin has a special affinity to calcium in bones and has therefore been used diagnostically/experimentally in studies of growth of bone weave in humans and animals.

The fish may be marked one or more times during its life. Because the fish is grows after marking, the placement and size of the mark will provide information regarding the time of marking relative to the age of the fish. If the fish is marked several times, there will be a corresponding number of marks in the calcium rich structures of the fish. For detection/proving of compounds absorbed/deposited in fish-scales, a mark (which will have the shape of a ring in fish-scales and otoliths) close the centre of the scale will indicate that the fish was marked at an early stage in life, while other marks closer to the edge of the fish-scale (i.e. having larger radius than the first mark) will represent marks made at a later time. The time for marking a fish might, for example, be every time it is transferred to a new producer, or a new net cage, so that the fish receives the marks as it is transferred.

The fish might also be marked just once, which will be the most appropriate. In these cases, the combination of the different compounds and/or derivates in the single mark correspond to a given course of events, and the history of the fish may thus be decided. At the time of marking, the prior and the future history of the fish are both stated. There will thus be cases where fish from one smolt producer is marked with different combinations of compounds/derivates, all identifying the same smolt producer, but not the same fish farming plant, because the fish could have been transferred to a number of production plants. In this case, the fish might be marked simultaneously at for example, vaccination.

The different compounds and/or derivates in each mark, and the number of marks and the sequence of these, will offer a large number of combinations. Each combination corresponds to a given course of events, and the history of the fish may thus be determined. This means that a fish with a mark containing a said derivate of alizarine complexone, will not have the same history as a fish with a mark containing another derivate of alizarine complexone, neither a third fish with a mark containing a combination of alizarin complexone and calcein. Derivates might also be achieved by using deuterium, as one or more deuterium atoms can be placed in specific positions on the compound being used for marking.

In addition to the combination of a number of compounds and derivates, and in cases where the fish is marked several times, the order of the marks, the amount of the different compounds might also be used to obtain a achieve a further differentiated mark.

Fish of all sizes might be marked in conventional ways. For small fish (eggs, larva and juveniles) the marking compound might for example, be added to a water bath, while for larger
fish the marking compound can be applied by intraperitoneal injection. If the fish is marked on the eyed eggs, larva or at early juvenile stages, the mark will only be incorporated in the otolith, because the fish-scales will not yet have developed. Such marks can, of course, not be detected without killing the fish. The marking of the larger fish might advantageously be preformed at the same time as other injections, such as vaccinations. One can also think of other embodiments where the compound(s) is(are) a component in a feed, so that the fish absorb the compound(s) through the gastrointestinal system.

There is an advantage to mark the fish at an early stage, but after the fish-scales are made, so that the fish is always marked. It will then be possible to separate farmed fish from wild fish, irrespective of whether the farmed fish escaped from the fish farm- or smolt plant. A further advantage of marking fish simultaneously with vaccination, is that the marking will not require separate individual handling of every fish.

All compounds used for marking fish according to the present invention, must be attached to or absorbed/deposited in calcium rich structures in the fish, and stay there for at least a substantial part of the fish's life, without substantially changing the metabolism of the fish. Also, the compounds must not exist naturally in the environment of the fish, as they can be absorbed in the calcium rich structures without control, and thereby destroy the commercial marking. The marks should of course not change the metabolism of the fish substantially, as the fish should be produced and utilized in the same way as today.

At least one of the compounds being used should be easy to detect. It might for example be a fluoridizing compound, or a compound having a colour, inside or outside of the visual area. When one decides whether a fish have been marked, with so-called gross detection, the discovery of such a compound in a calcium rich structure is sufficient to classify the fish as marked. If further information about the fish is desirable, one or more calcium rich structures can be high grade detected, and eventually other compounds in the calcium rich structures can be traced. As mentioned, the combination of the compounds and the amount ratio between them, provides the information available for the fish in question, by using a summary stating which groups of fish have been given which marks.

It might also be possible to use compounds which are changed/derived in a known way when absorbed in the calcium rich structures. The fish will, in such cases, be marked with one compound, while a derivate is detected / traced at a later stage.

In addition, at the least one of the compounds being used should be easy to derivate, so that a sufficiently differentiated mark-code can be achieved, as both the compound and its derivates can be used. Thus, it might be possible that one producer uses one compound for marking of
fish, but that different plant of this producer use different derivates of the compound. The compound will therefore identify the producer, while the derivate identifies the plant where the fish is produced.

In practice, every fish belongs to a group, and all the fish in the group have the same history. Every fish in the group is marked with one or several chemical compounds, as both the compound(s) and the amount of the different compounds are equal for every fish in the group, and is reserved for that group. The groups can thus be identified from which compounds and/or the amount of compounds, that are deposited in the calcium rich structures of the fish. Of course, it is necessary that there is a summary stating which groups are marked with which compounds, and the history of each group.

Since the compounds also are fastened to/absorbed/deposited in calcium rich structures being easily available and removable without hurting the fish, for example external fish-scales, it is possible to carry out the whole detection and decide where the fish came from, without having to kill it, and even after it is gutted, meaning after entrails, head and tail fin have been removed. It can also be decided from a fillet, if the skin has not been removed.

There is many different compounds which can be used according to the present invention, see for example H. J. Conn's Biological Stains, Williams & Wilkins Co, Baltimore, 9th ed. 1977, page 341. The compound can be chosen from the group comprising Basic Fuchsin, Brilliant Blue G, Brilliant Cresyl Blue, Eosin, Eosin Yellow, Methyl Violet B, Ponceau S, Nuclear Fast Red, Hemaoyxin, Erythrocin B, Basic Blue 24, Calcein Blue, Oxytetracycline, Alizarin Red S, Rodizonic Acid, Xylenol Orange, Alizarin Violet 3R, Alizarin Yellow GG, Alizarin Cyanin Gr. G, Alizarin Yellow R, Indigo Carmine, Flourescein, Fast Green FCF, Chlorazol Black E, Remazol Brilliant Blue, Alizarin Complexone, Calcein, and Procion Brilliant Blue, and derivates of these.

Example

The present invention will, in the following, be described with reference to experiments for marking of fish. The results are given in the accompanying figures, where

Figure 1 shows fluorescent marks in fish-scales at different times during the experiments,

and

Figure 2 shows the results of high grade detection of fish-scales, at different times during the experiments.
Two experiments for marking of fish were performed, the first one was a short term experiment with sampling and evaluation of changes during a period of time. The experiment had a duration of 14 days, and involved six groups of fish, wherein five were marked with different concentrations of alizarin complexone, and one group, the control group, remained unmarked. It was only registered death-rate at the highest concentration (150 mg/kg) after one day, when one fish died, corresponding to 20%.

In the short term experiment, a macroscopic examination of pancreas was also performed, to map potential histology changes due to the marking, from all groups after 14 days. Three fishes from each group were examined. The results showed no cell changes in any of the experiments, regardless of the concentration.

The second experiment was a long term experiment, lasting 6 months, wherein two concentrations of alizarin complexone were used for marking the fish; 100 mg/kg as a relative high dose and 30 mg/kg as a relative low dose. Necessary samples for qualitative and quantitative examination of outer structures, were taken and processed period of the project.

Alizarin complexone (ALZ) [Sigma A3882; C_{19}H_{16}NO_{6}; FW 385.3; Lot 117H0509; 3952-78-1] was used as the active marking compound in both experiments, as alizarin complexone is regarded as a model compound for the compounds of interest in relation to the present invention. The marking compound was dissolved in physiological saline, to which was added NaCl and buffered by adding tris-buffer to a pH close to 7. NaCl added at the rate of 300 mg NaCl per 100 ml of physiological saline. Table 1 provides a summary of weighted alizarin complexone and measured pH values in the solutions.

![Alizarin Complexone](image)

The fish were marked by injection in the abdomen. The fish in the short term experiment was presmolt salmon of even size and an average weight of 22.5 g. In the long term experiment, salmon having an average weight of 30 g was used. All fish were injected with a standard fluid
volume of 0.2 ml. The control group (0 mg/kg) was injected with 0.2 ml physiological saline. The fish was fin-/jaw-cut at the same time, according to S-1011.

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<th>Table 1</th>
<th>Summary of experimental groups, number of fish, concentration of alizarin complexone and other relevant data and information in relation to the experiments</th>
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<td>Group</td>
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<td>Short term experiment</td>
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</tr>
<tr>
<td>Number of fish</td>
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</table>

**Gross detection - qualitative detection in outer structures**

In order to decide whether the fish was marked, gross detection of fish-scales was used. Scales have a relative clean and even surface where the growth is mainly two-dimensional, making the scale well suited for fluorescence/colour measurement. It is already an established routine among anglers and others handling fish, to withdraw scale samples, and this renders scales even more attractive as a measuring object.

In a conventional fluorescence metre, it can easily be established whether the fish is marked with a fluorescing compound. It can also be used compounds not being fluorescing, but having a colour in or outside of the visible area. In those cases where it is used a compound
having a colour, a spectrometer might, for example, be used to trace the compound to establish that the fish is marked. Intraperitoneal marking showed good dyeing in teeth, fin rays and scales, and the emission/fluorescence was virtually equal from all calcium rich structures.

The gross detection was performed by scales from the different fish at the different times being dried and attached to a plastic strip, which then were placed diagonally in a cuvette, following which cuvettes were placed in a spectrometer. The fluorescence or colour was then measured in a known manner by the spectrometer.

A modified gross detection was also performed, wherein the fish-scales were treated as above, but a liquid was added to the cuvette, in addition to the plastic strip containing the scale. The pH value of such a liquid influenced the strength of the measured signal, wherein acids weakened the signal. Thus, for example, the emission from a scale might first be measured regularly without any liquid or with a base, and then with an acid, so that the emission from the latter may be used to deduct all unspecified emission from the scale. In this way even weak signals may be detected and used to trace the compound in question.

Other methods which will be apparent for a person skilled of the art, may, of course, be used for gross detection, for example microscoping.

The mark was easily seen in all samples from the short term experiment, except group 1 (10 mg/kg) which returned a marginal value for safe detection. Regarding the fluorescence, optimum excitation was achieved at excitation light about 530 nm, and the emission was maximum around 620 nm.

Marking with 30 mg/kg alizarine complexone gave a visible mark, but seemed to be to weak to return an unambiguous reading of scales, after 3 and 6 months. However, marking with 100 mg/kg returned a mark which was detectable during the whole period of the experiment. The strength of the mark was reduced during the period of the experiment, and the measurements indicated a halving of fluorescence during six months. This might, among others, be related to the growth structure of scales. The growth in scales is in three dimensions, even if the growth in the two-dimensional plane dominates. Reduction in emission may be due to changes in surface structure and increased thickness, increased absorption and scattering, thereby reducing the strength of the signal back to the measuring apparatus (reduced emission). However, visual inspection of scales in a fluorescence microscope showed that marks both in high- and low dose groups were easily seen after six months (see Figure 1. The intensity can not be compared between the pictures due to automatic aperture adjustment in the camera used).

Experience with fluorescence marks in calcium rich structures in fish, until present, shows that the mark in the scale will be durable during the whole, or at the least substantially, part of,
the fish's life. Scales are metabolically inactive when they are first made, with the exception of possible erosion at the outer edge as a result of lack of calcium during particularly difficult food situations. The inner part of the scale that shows growth zones is covered by pigmented skinfolds on the outside, and will thus be protected from UV radiation which might possibly lead to excitation and weakening of the mark.

**High grade detection - quantitative detection in outer structures.**

Scales can be dissolved without essentially degradating alizarine complexone, and during the long term experiment the amount of alizarin complexone in scale samples from different time periods, were high grade detected. The samples were processed and completely dissolved in formic acid with 10 % water, at 80°C over 10 hours, and then evaporated to a volume of about 100 micro litre and transferred to a sample glass with 200 microlitres "low-volume insert". The samples were then analysed with LCMS (Liquid Chromatography Mass Spectrometry) on an Agilent 1100 MSD instrument. They could have been analysed on a GCMS (Gas Chromatography Mass Spectrometry) or another analytical method, as would be apparent to a person skilled of the art. The principle of Mass spectrometry has "fingerprint" qualities, and is a good starting point when creating a detection-system for marking, according to the present invention.

The LCMS-method is based on chemical ionization in negative mode with m/z 384 as the main ion. The samples were analysed by injection of 1 µl extract by means of an autosampler, and separated on a 15 cm cyanocolumn having an mobil phase of 80/20 methanol/ammonium-acetat 50 mM and mobilphase flow of 1 ml/min. Alizarin complexone, under these conditions had a retention time of 2,3 minutes and a total analyse time of 5 minutes per sample.

The results are shown in Figure 2. The results of the measurements for m/z 384 are given as area-units for a peak at a retention time of 2,3 min, and expresses the relative amount of alizarin complexone in the samples (M/z is a ration number between the mass of the molecule (molecule weight) and the number of charges the molecules have (usually 1)). Also shown are results for m/z 326 which may be an expression for a possible degradation product of alizarin complexone (alizarin complexone after loss of one of the carboxylic acid groups).

Alizarin complexone could be analysed at all measurement points, and the measurements of scales from fish marked with 100 mg/kg showed higher response than measurements from fish given 30 mg/ml (see Figure 2, m/z 384). A response reduction after 6 months was shown, probably due to a technical measuring artefact, and the sampling of scales being based on weight scale-material, and not on the number of scales.
Starting with the expectation that the quantum marking compound incorporated in scales will remain unchanged, the measurements show a relatively large variation between the experimental groups, but small variation within each group (Figure 2). The variation between the experimental groups may have several causes, among others that the amount scales used in the analyses were based on volume and not number of scales. This may have resulted because fewer scales were used at the analysis of samples from 6 months, than those from 1 and 3 months.

As will be understood by persons skilled in the art, the present invention is not limited to solely what is shown and described above. The invention also comprises combinations and sub-combinations of the described features, and modifications and variations of this, which will be obvious for a person knowing the state of the art, and which is within the scope of the following claims.
Claims:
1. Use of a chemical compound and/or derivate for commercial, traceable marking of fish, wherein the compound is chosen from a group comprising compounds not being naturally present in the fish's environment, and which durably binds to calcium rich structures in the fish without substantially changing the fish's metabolism, or combinations of these compounds, and wherein the compound or one of the compounds in the combination, administrated to the fish can be detected in calcium rich structures by colour of fluorescence, and wherein every fish is marked once with a compound and/or amount of compound being specific for a group of fish, so that the group to which the fish belong to can be identified without killing the fish.

2. Use according to claim 1, wherein the compound or combination of compounds and/or the amount of the compound(s) being specific for a group, can be identified from calcium rich structures of the fish, preferably fish-scales, by mass spectroscopy.

3. Use according to claim 1 or 2, wherein at the least one of the compounds can be derived.

4. Use according to anyone of claims 1-3, wherein the compound or compounds is chosen from the group comprising Basic Fuchsine, Brilliant Blue G, Brilliant Cresyl Blue, Eosin, Eosin Yellow, Methyl Violet B, Ponceau S, Nuclear Fast Red, Haeomxylin, Erythrosin B, Basic Blue 24, Calcein Blue, Oxytetracycline, Alizarin Red S, Rodizonic Acid, Xylenol Orange, Alizarin Violet 3R, Alizarin Yellow GG, Alizarin Cyanin Gr. G, Alizarin Yellow R, Indigo Carmine, Fluorescein, Fast Green FCF, Chlorazol Black E, Remazol Brilliant Blue, Alizarin Complexone, Calcein, and Procion Brilliant Blue, and derivatives of these.

5. Use according to claim 4, wherein only alizarine complexone and its derivatives is used.

6. Composition for commercial, traceable marking of fish, comprising a chemical compound and/or derivate, wherein the compound is chosen from a group comprising compounds not naturally present in the fish's environment, and which durably binds to calcium rich structures in the fish without substantially changing the fish's metabolism, and combinations of these compounds, and where the compound or one of the compounds in the combination being administrated to the fish can be detected in calcium rich structures by colour or fluorescence, characterized in that the compound or combination of compounds and/or the amount of the
compounds with which the fish is marked, is specific for a group of fish, so that the group to
which fish belongs can be identified without killing the fish.

7. Composition according to claim 6, characterized in that the compound can be identified from
calcium rich structures of the fish, preferably fish-scales, by mass spectroscopy.

8. Composition according to claim 6 or 7, characterized in that at the least one of the
compounds can be derived.

9. Composition according to anyone of the claims 6-8, characterized in that the compound or
compounds is chosen from the group comprising Basic Fuchsin, Brilliant Blue G, Brilliant
Cresyl Blue, Eosin, Eosin Yellow, Methyl Violet B, Ponceau S, Nuclear Fast Red, Hematoxylin,
Erythrocin B, Basic Blue 24, Calcein Blue, Oxytetracycline, Alizarin Red S, Rodizonic Acid,
Xylenol Orange, Alizarin Violet 3R, Alizarin Yellow GG, Alizarin Cyanin Gr. G, Alizarin
Yellow R, Indigo Carmine, Fluorescein, Fast Green FCF, Chlorazol Black E, Remazol Brilliant
Blue, Alizarin Complexone, Calcein, and Procion Brilliant Blue, and derivates of these.

10. Composition according to claim 9, characterized by comprising alizarine complexone and
its derivates.

11. Method for marking of fish, characterized in that a composition according to anyone of the
claims 6-10 is administerated to the fish.
Figure 1. The pictures show fluoridizing marks in fish-scales after marking with alizarin complexone at different doses and at different sampling-times. The intensity can not be compared due to automatic aperture adjustment in the camera being used. The mark is shown as a function of time and concentration in fish-scale.
Figure 2.
Quantitative analyses of alizarin complexone in fish-scales of fish sampled 1, 3 and 6 months after marking, by intraperitoneal injection of salmon juveniles.

Response (median, ± Std. Err) for m/z 384 and 326. The number of individuals are marked over each plot. M/z 384 represents the whole alizarin complexone, while m/z 326 represents a degradation product after one of the carboxylic acid groups are lost.
## INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

**IPC7:** A01K 61/00, A01K 11/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

**IPC7:** A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE, DK, FI, NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**EPO-INTERNAL, WPI DATA, PAJ**

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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* Further documents are listed in the continuation of Box C. * See patent family annex.

**Date of the actual completion of the international search**

**15 December 2003**

**Date of mailing of the international search report**

**17-12-2003**

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