Abstract: The invention provides compositions and methods for treating or preventing harmful algal blooms such as red tides. Algicidal compositions comprising powdered dried plant material of Syzygium aromaticum and of at least one plant selected from Myristica fragrans and Pimpinella anisum are described as well as methods for treating harmful algal blooms which comprise applying to the affected or susceptible waters an algicidal composition according to the invention.
METHODS AND COMPOSITIONS FOR THE CONTROL OF HARMFUL ALGAL BLOOMS

This invention relates to methods and compositions for the control of harmful algal blooms.

Algae are generally microscopic organisms and may be thought of as simple aquatic plants which do not have roots, stems or leaves and have primitive methods of reproduction. Some algae display primitive animal features such as motility, while blue-green algae differ markedly from plants and all other algae in that they have a cellular structure and function that is more common to bacteria than to the plant kingdom.

Algae live in a wide range of aquatic environments and are a natural component of most aquatic ecosystems. Aquatic algae are found in both fresh and marine waters. They range in size from those visible only under a microscope to large kelps which may be metres in length.

Rapid reproduction of microscopic aquatic algae, caused by certain conditions such as high nutrients combined with warm temperatures and good light, can result in a dense population of algae called a bloom, which is visible to the naked eye. Algal blooms may occur in freshwater as well as marine environments. Many types of algae form blooms, some of which are harmless or even important to the health of the ecosystem. However, some blooming organisms contain toxins, other noxious chemicals or pathogens which can be harmful to aquatic life, fish stocks and to human health. These harmful algal blooms (HABs) are sometimes referred to as "red tides" after the colour of the bloom in the water, although the colour of the bloom depends on the species present and not all HABs are red.

Red tides dominated by the harmful dinoflagellate Cochlodinium polykrikoides have caused annual losses of USD $5-60 million to the Korean aquaculture industry annually since 1995 and a loss of USD $3 million during a 1999 net-pen fish mortality event in Canada (Jeong et al. 2008). In Japan red tides mainly kill finfish and have frequently caused very severe fisheries damage.
of more than 100 million yen (about one million US$) (Fukuyo et al. 2002). The average economic loss associated with noxious red tides is around 1.5 billion yen per year; it is therefore important to predict occurrences of red tide containing noxious species which kill fish and bivalves in order to reduce the negative impact on the aquaculture industry (Imali et al. 2006).

Among the important red tide organisms, *Cochlodinium antiqua*, *Heterocapsa circularisquama* and *Cochlodinium polykrikoides* are rated as extremely harmful species that can easily reach the warning level of fishery damage by consuming only small amounts of nutrients (Imali et al. 2006).

*Cochlodinium polykrikoides* is an unarmoured, marine, planktonic dinoflagellate species with a distinctive spiral-shaped cingulum. It is a cosmopolitan species found in warm temperate and tropical waters (Steidinger and Tangen 1996). This species was first reported from the Caribbean Sea along the southern coast of Puerto Rico (Margalef 1961). It has since been reported in northern Atlantic waters along the American east coast: Barnegat Bay, New Jersey (Silva 1967), and the York River, Virginia (Ho and Zubkoff 1979; Zubkoff et al. 1979). It is widely distributed in northwestern Pacific waters along the coasts of Japan and Korea (Fukuyo et al. 1990; Kim 1998) associated with fish kills. The emergence of *Cochlodinium polykrikoides* blooms in the Peconic Estuary and Shinnecock Bay, NY, USA during 2002-2006 was reported. Bioassay experiments demonstrated that after 24 h exposure to bloom waters at cell densities >5 x 10^4 cells ml^-1 100% of multiple fish species were killed while fish survived for 48 h at cell densities below 1 x 10^3 cells ml^-1 (Gobler et al. 2008).

Recently red tide was also found in the northwestern coast of the United Arab Emirates and has affected large areas of coastline. The bloom has killed fish and coral, causing huge environmental damage as well as financial losses. Diving operators and hotels are suffering financially as are desalination plants because of the costs of changing water-intake filters (The National Newspaper, Abu Dhabi, 26 February 2009).
The algae are an athecate species, i.e. without the cal plates. Cells are small, oval and slightly flattened dorso-ventrally. Cells range in size from 30-40 urn in length to 20-30 urn in width (Silva 1967; Yuki and Yoshimatsu 1989; Fukuyo et al. 1990; Taylor et al. 1995; Steidinger and Tangen 1996).

It is a photosynthetic species with numerous yellowish-green to brown chloroplasts, rod-shaped or ellipsoid in shape. The nucleus is situated anteriorly in the epitheca. A red stigma is present dorsally in the epitheca (Silva 1967; Yuki and Yoshimatsu 1989; Fukuyo et al. 1990; Taylor et al. 1995).

The alga is a planktonic species. It is a common ichthyotoxic 'red water' bloom species in the northwestern Pacific. This species commonly forms cysts (Fukuyo et al. 1990; Taylor et al. 1995; Steidinger and Tangen 1996).

*Cochlodinium polykrikoides* is a known red tide species associated with extensive fish kills and great economic loss in Japanese and Korean waters (Yuki and Yoshimatsu 1989; Fukuyo et al. 1990; Kim 1998). However, the actual toxin principles have yet to be elucidated (Taylor et al. 1995). Ho and Zubkoff (1979) suggested that physical contact, not a released toxin, was the cause of oyster larvae (*Crassostrea virginica*) deformation and mortality during a *C. polykrikoides* red tide in the York River (Virginia, USA).

*C. polykrikoides* closely resembles two other *Cochlodinium* species: *C. helix* and *C. helicoides*. The degree of rotation of the cingulum and sulcus distinguish the former species from the latter two: a. the cingulum in *C. polykrikoides* makes 1.8-1.9 turns around the cell, while in *C. helix* it is two turns and in *C. helicoides* it is 1.5 turns; and b. the sulcus turns 0.8 times between the proximal and distal ends of the cingulum in *C. polykrikoides*, whereas it is 1 time in *C. helix* and 0.6 times in *C. helicoides* (Silva 1967).

Many countermeasures have been applied for harmful algal blooms (Fukuyo et al. 2002; Imali et al. 2006). The counteraction techniques are roughly divided into two categories, indirect and direct methods. Indirect methods are basically important as prevention of red tide occurrences on a long-term scale (Imali et al. 2006) and include the following measures:
1. Regulation
2. Laws for the regulation of water quality
3. Water pollution control law
4. Marine pollution prevention law
5. Regulation of agriculture chemicals
6. Remediation
7. Water quality: absorption of inorganic nutrients by algae
8. Bottom sediment quality: dredging, aeration, digging, applications of sand, clay, lime, purification by benthos (Capitella)
9. Fish culture technique
10. Using moist pellets as bait
11. Keeping the proper scale and density of fish
12. Emergency procedures
13. Transfer of net cages
14. Cessation of feeding

In addition many methods for the direct control of red tides have previously been attempted (Shirota 1989; Song et al. 2005; Imali et al. 2006). These are:

1. Physical method
2. Physical shock: ultrasonic waves, electric current, air bubbling
3. Collection: suction, filtration, collection and centrifugation of surface red-tide water
4. Aggregation and sedimentation: clay, iron powder, macromolecular flocculant
5. Chemical method
6. Chemicals: hydrogen peroxide, organic acids, surfactants
7. Chemical reaction: ozone evolution
8. Biological method
9. Bivalves: oyster, etc
IO. Zooplankton: copepods, ciliates, heterotrophic dinoflagellates, nanoflagellates
H. Algicidal bacteria
12. Algicidal viruses

For environment-friendly mitigation strategies for red tides, biological controls using diatoms and algicidal bacteria and viruses are proposed (Fukuyo et al. 2002). A new finding of the abundant existence of algicidal bacteria on the surface of seaweed suggests that co-culturing fish and seaweed is a prevention strategy for harmful algal blooms by virtue of the continuous release of many algicidal bacteria to the surrounding seawater. The artificial development of seaweed beds would also be effective as a prevention strategy for red tides (Imali et al. 2008). Recently the Strombidinopsis jeokjo (a naked ciliate) has been introduced to control C. polykrikoides in and effectively reducing natural populations of C. polykrikoides from approximately 1000 cells ml\(^{-1}\) to below 10 cells ml\(^{-1}\) within 2 days. The growth and ingestion rates of cultured S. jeokjo on natural populations of C. polykrikoides in the mesocosms for the first 30 h (0.72 day\(^{-1}\) and 51 ng C grazer\(^{-1}\) day\(^{-1}\)) were 84% and 44%, respectively, of those measured in the laboratory during bottle incubations with similar initial prey concentrations. The calculated grazing impact of S. jeokjo on natural populations of C. polykrikoides suggests that large-scale cultures of this ciliate could be used for controlling red tides by C. polykrikoides in small areas (Jeong et al. 2008).

Overall still the method suggested by Shirota (1989) represent one promising strategy that could be used to treat red tides with flocculants such as clay, which scavenge particles, including algal cells, from seawater and carry them to bottom sediments and implemented in Kyushu and Korea have been successful to some extent for the removal but not killing of C. polykrikoides red tides (Fukuyo et al. 2002). Thereafter, these chemical and physical control options have received little attention (Fukuyo et al. 2002). Accordingly, there are at present no wholly successful physical or chemical control methods (Imali et al. 2006).
Hence, there remains an urgent need for improved compositions and methods for the control of harmful algal blooms, said compositions and methods being safe and effective and without adverse effect on the environment.

Accordingly, the present invention provides a method for treating or preventing harmful algal bloom, which comprises applying to the affected or susceptible waters an effective amount of a composition comprising powdered dried plant material of Syzygium aromaticum and of at least one plant selected from Myristica fragrans and Pimpinella anisum.

In a preferred embodiment the composition comprises powdered dried plant material of Syzygium aromaticum, Myristica fragrans and Pimpinella anisum.

The present invention arises from the inventors' finding that simple, ground preparations of certain herbs exhibit a marked algicidal effect when tested against red tide seawater comprising the harmful algae Cochlodinium polykrikoides.

Syzygium aromaticum (syn. Eugenia aromaticum, Eugenia caryophyllata, Eugenia caryophyllus or Caryophyllus aromaticus) is a tropical tree, the immature, unopened flower buds of which provide, when dried, the spice known as cloves. Whole or ground cloves are a popular spice in North African, Middle Eastern and European dishes, both savoury and sweet. Cloves contain 15 to 20% essential oil which is mostly Eugenol, the principal flavour-giving volatile oil which is a very strong antiseptic. Clove oil is a strong stimulant and carminative and is traditionally used to treat nausea, indigestion and dyspepsia. It is also commonly used to treat toothache.

Myristica fragrans (nutmeg) is a culinary herb whose fruit gives us the spices: nutmeg (the seed kernel) and mace (the aril, or lacy covering on the kernel). Powdered or grated nutmeg is used to flavour savoury and sweet dishes in Asian, Middle Eastern and European cuisines. The essential oil is used as natural food flavouring in baked goods, syrups (e.g. Coca Cola), beverages, sweets etc. In traditional medicine nutmeg and nutmeg oil were used for illnesses related to the nervous and digestive systems. The essential oil, obtained by the
steam distillation of ground nutmeg, is also used in the cosmetic and pharmaceutical industries e.g. in toothpaste and cough syrup. Myristicin and elemicin are believed to be the chemical constituents responsible for the subtle hallucinogenic properties of nutmeg oil. Other known chemical ingredients of the oil are \( \alpha \)-pinene, sabinene, \( \gamma \)-terpinene and safrole.

*Pimpinella anisum* (anise, aniseed) is a culinary herb whose fruit is used for flavouring cakes, breads, fish, meat and vegetable dishes, as well as alcoholic drinks and cordials. Anise is also used in traditional herbal remedies e.g. to aid digestion, ease trapped wind, cure hiccups, freshen breath, soothe coughs, aid sleep and relieve toothache. Anise oil, whose principal component is anethole, is used to treat lice and scabies and, mixed with other ingredients, against insects. It is also reported to have antifungal and antiviral activities suggesting its use in the treatment of colds and ‘flu.

The results disclosed herein show that preparations of *S. aromaticum*, *M. fragrans* and *P. anisum* each show effective algicidal activity in a test assay using red tide seawater comprising *C. polykrikoides* as the dominant organism. The herb *S. aromaticum* appears to have the greatest activity in the test system. Synergistic effects are observed with combinations of two or more herbs such as *S. aromaticum* and *M. fragrans*, or *S. aromaticum* and *P. anisum*. The combination of *P. anisum*, *M. fragrans* and *S. aromaticum* provides particularly effective algicidal activity. In a preferred embodiment, the composition comprises *Pimpinella anisum*, *Myristica fragrans* and *Syzygium aromaticum* in a ratio of about 6:3:1.

As used herein, "algicidal" means the property of killing algae or arresting or retarding their normal growth and development such that a harmful algal bloom is prevented or ameliorated.

The ability of compositions as described herein to kill harmful algae is demonstrated in the following examples in a test system using seawater affected by "red tide" harmful algal bloom in which *Cochlodinium polykrikoides* is the dominant species, however, there is no reason to suppose that the algicidal activity will be restricted to only this species of harmful algae or this type of
harmful algal bloom. The methods and compositions of the invention are expected to have utility against any harmful algal bloom including, but not limited to, those caused by species of algae mentioned in the introduction and the examples.

Because compositions as described herein comprise preparations of plant origin, said plants being herbs already consumed by man, there is a low risk of toxicity to man or the environment from the use of said compositions for the treatment or prevention of harmful algal blooms.

The compositions described herein comprise powdered dried plant material e.g. the dry seed or kernel, or fruit or part thereof (e.g. aril), or bud, or flower, which has been ground to a powder. Suitable dry herb materials of the plant species described herein are readily available, e.g. dried flower buds (sometimes called pods) of *S. aromaticum*, seeds (nutmeg) or arils (mace) of *M. fragrans* and fruit of *P. anisum* (anise "seed" or aniseed). The foregoing substances are readily obtainable owing to their culinary use as spices. The plant material is ground to a powder to allow easy dispersal of the preparation and promote diffusion of the algicidal agent.

Clove flower (unopened flower buds of *S. aromaticum*) comprise two parts: the stem or stalk is composed of the calyx and terminates in four spreading sepals on which rests a small bulbous head formed by the unopened petals. Ground preparations of whole cloves, stalks and heads each exhibit algicidal activity against species present in HABs, however, the inventors have found that ground clove stalks show greater algicidal activity (faster killing of HAB cells) than ground clove heads. Accordingly, in a preferred embodiment, the plant material of *S. aromaticum* is clove stalks, i.e. a preparation of cloves which is substantially free of clove heads. It will be appreciated that it may not be practicable or economic to obtain pure clove stalks from a batch of whole cloves; it may therefore be sufficient for practical purposes to enrich a batch of whole cloves for stalks by removal of the majority of, or a significant proportion of, the heads prior to milling.
Nutmeg is the seed of *M. fragrans* and comprises a nucleus or kernel surrounded by a sheath or coat. The inventors have found that the algicidal effect is confined to the nutmeg seed kernel. Accordingly, in a preferred embodiment, the plant material of *M. fragrans* is nutmeg seed kernel from which the seed coat has been substantially removed prior to milling to yield a preparation having even greater algicidal activity than that of ground whole nutmeg.

The above mentioned plant parts have been identified by the inventors as having profound algicidal effects, however, other plant parts may be used in the compositions and methods of the invention. The observed algicidal effects will be brought about by one or more active agents produced by the respective plant. These active agents may be found in other parts of the plant to those specifically mentioned above. Any plant part containing the active agent(s) is a candidate material for preparing an algicidal composition according to the invention. Plant parts containing the active agent(s) may be determined empirically by measuring algae killing or inhibitory activity in a test assay such as the one described herein. Alternatively, once the active agent(s) has been identified, the concentration of the active molecule(s) in different tissues or organs of the plant can be measured directly. For practical purposes, preferred plant parts are those which accumulate the active ingredient in relatively large amounts. Ideally, the plant part is relatively easy to harvest and handle, such as a seed or fruit. However, other parts such as leaves, roots, stems or flowers or parts thereof may be suitable provided that the active agent(s) is present in sufficient quantities to give the desired algicidal effect. Conveniently, the herbs may be cultivated according to normal agricultural practices and the plant part containing the active agent(s) harvested and dried. The dried plant material can be used in the algicidal compositions and methods of the invention with minimal processing: typically, the plant material is cleaned (as necessary) and milled (ground) to a powder. The inventors have found that by milling the plant material twice a fine powder can be produced which is particularly efficacious for killing HAB. Accordingly, in a preferred embodiment the composition comprises dried plant material which has
been ground to a fine powder e.g. by a preliminary milling to produce a preparation of coarse particles, followed by one or more further millings to produce a fine powder.

It may be desirable to purify the algicidal fraction of the herb preparation to a lesser or greater extent prior to use in a method or composition according to the invention.

The method of the invention may be applied to harmful algal blooms wherever they occur, including freshwater (e.g. rivers, lakes, ponds and reservoirs) and seawater (e.g. open sea, including coastal waters). Compositions according to the invention are conveniently applied to the surface of the water as a powder. A rate of 750 gm⁻² has been found to be effective in the control of HABs in the open sea.

Alternatively, superior algicidal effects can be achieved by delivering the herbal powder below the surface of the affected waters e.g. in the region 0.5 to 1.0 m below the surface. By this method, an application rate of 150 gm⁻² may be sufficient for control of HAB in open water.

In another embodiment, the invention provides the use of a composition comprising powdered dried plant material of *Syzygium aromaticum* and of at least one plant selected from *Myristica fragrans* and *Pimpinella anisum* for the treatment or prevention of harmful algal bloom.

In a further embodiment, the invention provides method for killing *Cochlodinium* spp., which comprises treating the organism with a composition comprising powdered dried plant material of *Syzygium aromaticum* and of at least one plant selected from *Myristica fragrans* and *Pimpinella anisum*. Preferably the *Cochlodinium* sp. is *Cochlodinium polykrikoides*.

In a still further embodiment, the invention provides a method for disinfecting/decontaminating vessels, such as ships and boats, and other marine tools, instruments and facilities (offshore or onshore) contaminated with harmful algal bloom, which comprises applying to the vessels, tools, instruments or facilities a composition comprising powered dried plant material of *Syzygium*
aromaticum and of at least one plant selected from Myristica fragrans and Pimpinella anisum.

In yet another embodiment, the invention provides an algicidal formulation comprising powdered dried plant material of Syzygium aromaticum and of at least one plant selected from Myristica fragrans and Pimpinella anisum, and one or more vehicles, carriers, binders or excipients. The algicidal formulation is useful for the treatment and prevention of harmful algal blooms, e.g. red tides, in the open sea, including coastal waters. The algicidal formulation has further utility for the disinfection/decontamination of vessels and other marine tools, instruments and facilities contaminated with harmful algal bloom.

Preferred features of different embodiments of the invention are as to each other mutatis mutandis.

The invention will now be illustrated by the following non-limiting examples which make reference to the figures, of which:

Figure 1 is a graph showing the effect of 0.3% suspensions of single milled (D) or refined milled (Δ) dried seeds mixture comprising 6 volumes of Pimpinella anisum, 3 volumes of Myristica fragrans and 1 volume of Syzygium aromaticum on the percentage killing of 8 species of HAB (Cochlodinium polykrikoides, Gymnodinium catenatum, Dinophysis caudate, Prorocentrum micans, Pyrodinium bahamense, Ceratium furca, Karenia mikimotoi and Protoperidinium spp.) in filtered seawater sample. Control (0) sample was not exposed to herbal powder.

Figure 2 is a graph showing the effect of 0.3% suspensions of refined milled herbal mixtures of formula one (see Fig. 1: 6 volumes of Pimpinella anisum, 3 volumes of ground whole Myristica fragrans and 1 volume of ground whole cloves Syzygium aromaticum) and formula two (6 volumes of Pimpinella anisum, 3 volumes of Myristica fragrans kernel and 1 volume of Syzygium aromaticum cloves stalk) on the percentage killing of 8 species of HAB (Cochlodinium polykrikoides, Gymnodinium catenatum, Dinophysis caudate, Prorocentrum micans, Pyrodinium bahamense, Ceratium furca, Karenia mikimotoi and Protoperidinium spp.) in filtered seawater sample.
Figure 3 is a diagram of the submerged "splashing gun" apparatus used to deliver refined milled seeds mixture from a boat to HAB affected area of open sea. The diagram shows, from top of page, rear, side and top views.

Figure 4. Curve showing the killing rates after splashing of 150g per square meter (m²) of refined milled dried seeds powder mixture comprising 6 volumes of Pimpinella anisum (95% purity), 3 volumes of Myristica fragrans kernel and 1 volume of Syzygium aromaticum cloves stalk on 30m² open seawater affected areas with 8 species of HAB (Cochlodinium polykrikoides, Gymnodinium catenatum, Dinophysis caudate, Prorocentrum micans, Pyrodinium bahamense, Ceratium furca, Karenia mikimotoi and Protoperidinium spp.). Three applications were made at 30 minute intervals.

EXAMPLE 1
MATERIALS AND METHODS

Preparation of a Homogenized Suspension of Red Tide

Seawater was collected from different locations with "red tide" of algae that is devastating the waters off the coast of Fujairah. Samples of 500 Litres of the red tide seawater were equally distributed into transparent plastic fish tanks attached with oxygen distributors.

The microscope examination method has shown the presence of eight different algae species that are found in the north western coast of the United Arab Emirates seawaters; these are Cochlodinium polykrikoides, Karenia mikimotoi, Prorocentrum micans, Dinophysis caudate, Pyrodinium bahamense, Gymnodinium catenatum, Ceratium furca and Ceratium fusus. In average the seawater contained approximately >7 x 10⁴ cells ml⁻¹ of the red tide in which the Cochlodinium polykrikoides is the most dominant species.

Herbal preparation

Dried clove pods Syzygium aromaticum, Pimpinella anisum L. seed, and Myristica fragrans seed were purchased from a local Dubai herbal market. All
herbal materials were cleaned from dust by being air-dried for 15 min in metallic strainers and were then powdered individually in a grinder.

Anti-Red Tides Assays

Different concentrations (WA/) of herbal formulas compositions comprised from *Pimpinella anisum*, *Myristica fragrans* and *Syzygium aromaticum* were prepared in 500 Litres seawater and 500 Litres control sample (herbal material not added) as follows:

Formula one:

*Pimpinella anisum* 0.3%; and *Myristica fragrans* 0.2% 

Formula two:

*Syzygium aromaticum* 0.5%, *Pimpinella anisum* 0.3% and 0.2% of *Myristica fragrans*.

After incubation for 1h at 25°C the numbers of red tides cells were checked microscopically and recorded for each sample.

RESULTS

The tests of seawater have shown the presence of eight different algae species that cause red tide these are *Cochlodinium polykrikoides*, *Karenia mikimotoi*, *Prorocentrum micans*, *Dinophysis caudate*, *Pyrodinium bahamense*, *Gymnodinium catenatum*, *Ceratium furca*, *Ceratium fusus*. The red tide of *Cochlodinium polykrikoides* is the most dominant species on the rest, and continues to spread in the waters of the northwestern coast of the United Arab Emirates.

The results set out in Table 1 indicates that each aqueous solution of 0.3% of *Pimpinella anisum* seed, *Myristica fragrans* seed and clove pods *Syzygium aromaticum*, powder has red tidecidal activity alone in comparisons to untreated control sample.
The combination of *Pimpinella anisum* 0.3% and *Myristica fragrans* 0.2% showed a profound synergy against red tide harmful algal bloom (Table 1). Moreover, the addition of clove pods at concentration 0.5% to the combination 0.3% *Pimpinella anisum* and 0.2% *Myristica fragrans* appears to have a significant beneficial effect on the percentage killing of the red tides algae (Table 1). Furthermore, the aqueous solutions >0.5% clove pods has specific anti-red tides activity in the sample under treatment.

The role of the clove pods in interaction with both *Pimpinella anisum* and *Myristica fragrans* seed needs further investigation.

Table 1. The percentage of killing (number of non-motile lysed cell per 100 counted cells) of *Cochlodinium polykrikoides* and other red tides algae in aqueous solutions prepared in seawater of different herbal compositions measured at various intervals of incubation at 25°C.

<table>
<thead>
<tr>
<th>Percentage of aqueous solutions of herbal materials</th>
<th>1 min</th>
<th>5 min</th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Pimpinella anisum</em> 0.3%</td>
<td>5%</td>
<td>30%</td>
<td>80%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><em>Myristica fragrans</em> 0.3 %</td>
<td>5%</td>
<td>30%</td>
<td>78%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><em>Syzygium aromaticum</em> 0.3%</td>
<td>50%</td>
<td>80%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Formula One: <em>Pimpinella anisum</em> 0.3%; and <em>Myristica fragrans</em> 0.2%</td>
<td>15%</td>
<td>50%</td>
<td>85%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Formula Two: <em>Syzygium aromaticum</em> 0.5%, <em>Myristica fragrans</em> 0.2% and <em>Pimpinella anisum</em> 0.3%</td>
<td>60%</td>
<td>90%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
EXAMPLE 2
MATERIALS AND METHODS

Direct treatment of HABs in an open sea

A location of seawater devastated with HABs has been identified on the coast of Fujairah. A twenty square meter area was marked by 4 small boats. The microscope examination method was carried-out to identify the algae species on the surface of seawater and at depth of 1 and 2 meters. Seawater temperature was recorded.

Anti-Red Tides Assays

Formula 2, comprised from Syzygium aromaticum 0.5%, Myristica fragrans 0.2% and Pimpinella anisum 0.3%, showed the greatest activity against red tides algae in the 500 Litre tank test (Example 1). Accordingly, for the experiments in open sea, a dry herbal mixture was prepared in which the component herbs were present in the same ratio as in formula 2. Dried, powdered herbs as previously described were mixed together in the following amounts: 10 kg S. aromaticum, 4 kg M. fragrans and 6 kg P. anisum. Preliminary experiments were performed to determine an appropriate rate of application. For the experiment reported here, the dry herbal mixture was evenly scattered as a powder on a surface of seawater at rate of 750 g per 1 square meter. After allowing the powdered herbal mixture to be distributed in the seawater for one minute, samples of 10 ml of the seawater were examined and numbers of red tides cells were checked microscopically and recorded.

RESULTS

The microscope examination method has shown the presence of eight different algae species in the 20 square meters of seawater these are Cochlodinium polykrikoides, Karenia mikimotoi, Prorocentrum micans, Dinophysis caudate, Pyrodinium bahamense, Gymnodinium catenatum, Ceratium furca and Ceratium fusus in average of approximately >7 x 10^5 cells
ml⁻¹ in which the Cochlodinium polykrikoides is the dominant species and it was found at 1 x 10⁵ cells ml⁻¹. However, the number of algae species on the surface of seawater was approximately >7 x 10⁵ cells ml⁻¹, meanwhile, the number of algae was reduced gradually to 1 x 10³ cells ml⁻¹ and 1 x 10² cells ml⁻¹ at depths 1 and 2 meters, respectively. The seawater temperature was 26.8 °C ± 2.

The results set out in Table 2 indicate that there is 3 log reduction of HABs obtained after 2 min on surface of the seawater after the addition of herbal mixture formula 2 comprised from Syzygium aromaticum 0.5%, Myristica fragrans 0.2% and Pimpinella anisum 0.3%. Whilst there are only 2 and 1 log reduction in cells of HABs at 20 and 50 cm, respectively. This was probably due to normally water flux from the bottom. The results suggest that the higher death occurred at seawater surface.

Furthermore, it was noticed after the addition of powdered herbal mixture the clarity of seawater had increased for few seconds. On the other hand, some floated herbal mixture drifted for 100 meter from the treated area; this was probably due to normally water flow, however, the number of HABs reduced by 95%.

**Table 2.** Survival (cells ml⁻¹) of Cochlodinium polykrikoides and other red tides algae in open seawater treated with 75Og of formula 2 (Syzygium aromaticum 0.5%, Myristica fragrans 0.2% and Pimpinella anisum 0.3%) per 1 square meter.

<table>
<thead>
<tr>
<th>Depth of seawater from surface (cm)</th>
<th>Cells ml⁻¹ of HABs in seawater After 2 min from the addition of formula 2</th>
<th>Control untreated 1 square meter of seawater</th>
</tr>
</thead>
<tbody>
<tr>
<td>At the surface</td>
<td>&gt;1 x 10² cells ml⁻¹</td>
<td>&gt;7 x 10⁵ cells ml⁻¹</td>
</tr>
<tr>
<td>20 cm</td>
<td>&gt;1 x 10¹ cells ml⁻¹</td>
<td>&gt;7 x 10⁵ cells ml⁻¹</td>
</tr>
<tr>
<td>50 cm</td>
<td>&gt;7 x 10⁰ cells ml⁻¹</td>
<td>&gt;7 x 10⁵ cells ml⁻¹</td>
</tr>
</tbody>
</table>
CONCLUSION
The results confirm that the *Pimpinella anisum*, *Myristica fragrans* and *Syzygium aromaticum* anti-harmful algal bloom agent is an environmentally safe, target-specific product for controlling red tides in wildlife mortalities among marine and coastal species of fish, birds, marine mammals and other organisms.

None of the herbs *Pimpinella anisum*, *Myristica fragrans* and *Syzygium aromaticum*, nor any other herbal material, has been investigated previously in the literatures as anti-red tides. It appears that all herbs used in this invention have shown as effective anti-red tides in which *Syzygium aromaticum* has shown strong anti-red tides activity.

The red tideicide activity of *Pimpinella anisum*, *Myristica fragrans* and *Syzygium aromaticum* has been further studied in the open sea and shows to be an effective, fast-acting method to be used to treat HABs. The maximum reduction of HABs has occurred at the seawater surface despite the presence of normal water flow and surface wind speed. Because these algae are phytoplankton, i.e. autotrophic, they require light for photosynthesis and therefore live near the water's surface which is where blooms form. The algicidal compositions of the invention can therefore be effective in the control of HABs when applied to the water's surface.

Algae-toxic compounds of the *Pimpinella anisum*, *Myristica fragrans* and *Syzygium aromaticum* plants will be investigated. We are assuming that the modes of action of these compounds are complex, and a number of mechanisms in relation to red tides management are yet to be fully explored.

EXAMPLE 3
Materials and Methods
*Isolation of harmful dinoflagellates*

The species of harmful dinoflagellates *Cochlodinium polykrikoides*, *Gymnodinium catenatum*, *Dinophysis caudate*, *Prorocentrum micans*, *Pyrodinium bahamense*, *Ceratium furca*, *Karenia mikimotoi* and *Protoperidinium spp.*, used in this experiment were isolated from Dibba, coastal region at the
northeastern tip of the UAE on the Gulf during the winter (December to March of 2009) using a 25cm diameter, 20mm mesh plankton net Model 23.000KC (Denmark A/S). Plankton samples were screened gently through 20mm Monodur Nytal (Nylon KC, Denmark A/S) and placed in 11 glass bottle. After 2h the upper three fourths of the volume was gently discarded and the remaining volume transferred into 250ml polycarbonate (PC) bottles. For identification, were examined under a stereomicroscope (Model SZ61 and BX41 Olympus, Japan) with digital camera model DP20 (Olympus, Japan). HAB were counted using Sedgewick-Rafter cell Model 21.015 (KC Denmark A/S) with a volume 1ml under bright field/phase contrast/ dark field microscope Model BX41, (Olympus, Japan). In average the seawater contained approximately >7 x 10^4 cells ml^-1 of the red tide in which the Cochlodinium polykrikoides is the most dominant species. All the isolates of 8 HAB species were used within 1h after the collection from the seawater to observe the HAB killing rates after the addition of milled or refined milled seeds of Syzygium aromaticum, Pimpinella anisum L., and Myristica fragrans.

**Herbal preparation**

Dried intact clove pods Syzygium aromaticum, Pimpinella anisum L. seeds, and intact Myristica fragrans seeds were purchased from a local Dubai herbal market. All herbal materials were cleaned from dust by being air-dried for 15 min in metallic strainers and were then powdered individually in a grinder. To produce a very fine powder with small particles of each herbal seeds were refined and milled once more.

Clove (unopened flower buds of Syzygium aromaticum) are composed of two parts: the stem and a small bulbous head in the centre. The stem and the bulbous head were individually refined and milled twice for further investigations.

**Anti-HAB assays**

The anti-HAB assays were carried out in herbal suspension as follow: The herbal powders were added on 100ml of filtered seawater containing 8 mixed
species of HAB at a titer of >7 x 10^4 cells ml^-1. The controls were used the same 8 HAB species isolate that were not exposed to seeds powder. Survival was monitored with interval of 1 min for up to 10 min then every 5 min for up to 60 min by cell density under a BX 50 Olympus optical microscope.

Results and Discussion

Killing curves of 8 mixed HAB species after the addition of 0.3% w/v refined milled dried seeds mixture comprising 6 volumes of Pimpinella anisum, 3 volumes of Myristica fragrans and 1 volume of Syzygium aromaticum as shown in Figure 1: the number of live HAB cells were reduced rapidly in which 46% of the cells were killed within 2 min and no live cells were found after 6 min with the linear regression of R^2 value of 0.969. In comparison in the normal single milled seeds powder the HAB cells survived longer, whereas, 47% of live cells were reduced within 10 min and obtained 100% killing at 25 min with less liner regression value (R^2=0.8793) Fig 1.

It appears when the seeds can include further cracking or cutting step and then the broken seed is further milled or refined to produce a fine mixture with small particles the algaecidal activity increases. The linear regression to determine of R^2 values shows that refined milled seeds give higher value (R^2=0.969) than that of a normal single milled seeds (R^2=0.8793) Fig 1). Since, the mechanical cracking releases some of the oil which can’t be transferred or released into the seawater at 20°C, thereby seeds oil can't be considered as a killing factor on the red tides cells. It seems the powder texture of the product to which the particles size of the product has reduced, whereas, HAB killing efficiency has increased to give 100% killing (with R^2 = 0.969) on the red tide in seawater within 6 min (Fig. 1) comparing to that powdered of seeds milled once (normal milled) give 25 fold longer time to yielded 100% killing (R^2=0.8793) of HAB.

In general, the linear regression equation was used to predict the HAB percent killing. The correlation coefficient and regression index for algaecidal of refined milled seeds (Fig 1) assay at 1 to 7 min were all above 0.9 indicating a
very high linear relationship between the measured time and the actual killing of HAB. Therefore, the refined milled seeds have been adopted in the experiments. It was found that the single milled seeds $r$ was 0.8478 and for refined milled seeds was highest 0.969. This provided extra evidence for the significant and strong evidence for the reliability to use refined milled seed of the present invention to enhance the algaeidal activity.

Table 3. The percentage of killing (number of non-motile lysed cell per 100 counted cells) of 8 species of dinoflagellates (*Cochlodinium polykrikoides*, *Gymnodinium catenatum*, *Dinophysis caudate*, *Prorocentrum micans*, *Pyrodinium bahamense*, *Ceratium furca*, *Karenia mikimotoi* and *Protoperidinium* spp.) in various concentrations (w/v) of refined milled dried seeds mixture comprises one volume of each *Pimpinella anisum*, *Myristica fragrans* and *Syzygium aromaticum* prepared in filtered seawater measured at various intervals of incubation at 20°C.
Table 3

Percentage of suspensions of refined milled dried seeds powder

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0.1%</th>
<th>0.3%</th>
<th>0.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Refined milled dried seeds formulas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aromaticum</td>
<td>22</td>
<td>92</td>
<td>100</td>
</tr>
<tr>
<td>M. fragrans</td>
<td>13</td>
<td>29</td>
<td>52</td>
</tr>
<tr>
<td>P. anisum</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>S. aromaticum, M. fragrans (1:1)</td>
<td>100% within 4min</td>
<td>100% within 1.5min</td>
<td>100% within 1min</td>
</tr>
<tr>
<td>S. aromaticum, P. anisum (1:1)</td>
<td>20</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>M. fragrans, P. anisum (1:1)</td>
<td>6</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>S. aromaticum, M. fragrans, P. anisum (1:1:1)</td>
<td>30</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
Referring to Table 3, it shows that the refined powder of *S. aromaticum* has killed all 8 species of HAB cells almost instantly around 1 to 1.5 min in suspensions of 0.5% and 0.3%, respectively, whilst the killing efficiency has decreased to 92% with the 0.1%, which indicated that the refined powdered of the seeds of *S. aromaticum* is perfect red tidecidal.

From preliminary experiments, surprisingly, the intact dried clove pods *Syzygium aromaticum* added in seawater containing the 8 HAB at concentration of 0.5% gives 100% killing on the red-tide within 4 min. Again this indicates that the killing of HAB by *Syzygium aromaticum* is not oil dependence.

In the sample containing only the refined milled powder seeds of *Myristica fragrans* the maximum HAB killing rate yielded after 5, 10 and 15 min in the suspensions of 0.5%, 0.3% and 0.1%, respectively (Table 3).

In the case of *Pimpinella anisum* the HAB killing rates has increased gradually to reach >50% after 25 min in the powder suspension of 0.5% (Table 3). It is noteworthy to mention that we noticed the refined milled powder of *Pimpinella anisum* has the ability to move, scatter and distribute rapidly (few seconds) on the water surface and float for a few hours (2-3h). Samples of floated powder was collected and examined under microscope. The test has shown the small particles size of the *Pimpinella anisum* seeds powdered had trapped and arrested a large number of HAB and this could attribute to cause a retarding of their normal growth of what led to paralyze them from the movement which may explain why it takes about 20 min to kill HAB cells with a 0.5% suspension in comparisons to the *Syzygium aromaticum* and *Myristica fragrans* (Table 3).

This novel physical factor of the refined milled powder of *Pimpinella anisum* was used in the present invention for rapidly trap and arrest large number of HAB cells with combinations of *Syzygium aromaticum* and or *Myristica fragrans* to enhance the killing rates of HAB.

According to the embodiment above, it has been proved that the refined milled powder of *Pimpinella anisum* with *Syzygium aromaticum* and *Myristica fragrans* mixture were combined together to examine how the inhibition efficiency
affected by the different suspensions concentrations could inhibit the algae
growth to nil within only 1 min, 3 min and 5 min in the powdered suspensions of
0.5%, 0.3% and 0.1%, respectively (Table 3). Basically, the whole process was
the same within 1 to 5 min as mentioned above (Table 3). The invention shows
that there is a synergy between the refined milled powder of *Syzygium
aromaticum* and *Myristica fragrans* (ratio 1:1) was shown very clearly in the
suspension of 0.1 % in which the killing efficiency has increased to yield 100%
HAB killing within only 4 min in comparisons the HAB killing rates were achieved
within 10 min and 30 min with same suspension concentration (0.1 %) of
*Syzgium aromaticum* alone and *Myristica fragrans* alone, respectively (Table 3).
Moreover, while both *Syzygium aromaticum* and *Myristica fragrans* are added in
the suspension, the red tide growth is much more efficiently inhibited than that
only *Syzygium aromaticum* or *Myristica fragrans* or is added.

Accordingly, the result of the embodiment of the present invention shows
that *Pimpinella anisum* work synergistically with both *Syzygium aromaticum* and
*Myristica fragrans* for long-time lasting (3-4h) inhibiting the growth of HAB due to
the physical characteristic of the refined milled powder of *Pimpinella anisum* in
to ensure the condition of trapping and arresting a large number of HAB by diffusion
of the red tidecidal phyto activities on the surface of the object to thereby sustain
the red tide growth inhibiting effect of the inhibitor for a long period of time. In
addition, the red tide to which the HAB refined milled seeds powder growth
inhibitor has been applied are blighted and peeled away, and the HAB growth
inhibitor retards and weakens the growth of red tide and is therefore effective for
removing HAB. Still another characteristic of the HAB growth inhibitor comprises
of refined milled seeds powder of *Syzygium aromaticum* and or *Myristica
fragrans* and or *Pimpinella anisum* is that it is usable not damaging the surface
and the entire of the object to which it has been applied.

The effect of different parts segments of *Syzygium aromaticum* clove buds
and *Myristica fragrans* seeds on the killing rates of HAB.
The results from refined and milled powder of unopened flower buds of
*Syzygium aromaticum* stalk without bulbous head and bulbous head alone shows
that powdered stalk has killed all 8 species of HAB cells within 30 sec and 9 min with 0.3% and 0.1%, respectively, in comparison to powdered whole clove buds and bulbous head powder which is delayed by one and two minutes, respectively, to obtain 100 percent killing rates (Table 4).

Table 4. *Syzygium aromaticum* "clove" parts used to evaluate the percentage of HAB killing

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Percentage of suspensions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1%</td>
</tr>
<tr>
<td>Whole clove buds</td>
<td>30 50 80 93 98 100</td>
</tr>
<tr>
<td>Stem omitted bulbous head</td>
<td>40 65 90 96 100</td>
</tr>
<tr>
<td>Bulbous head alone</td>
<td>25 40 65 85 91 96 100</td>
</tr>
</tbody>
</table>

*Myristica fragrans* "nutmeg" consists of the outer sheath or coat and the inner nucleus or kernel. The HAB killing rates is also increased (killed in shorter time) with the refined milled inner nucleus alone in comparison to that obtained with refined milled whole seeds of *Myristica fragrans*, however, refined milled outer sheath alone has no killing effect on HAB (Table 5).

Table 5. *Myristica fragrans* "nutmeg" parts used to evaluate the percentage of HAB killing

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Percentage of suspensions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1%</td>
</tr>
<tr>
<td>Whole Nutmeg</td>
<td>31 55 72 88 96 100</td>
</tr>
<tr>
<td>Nucleus</td>
<td>37 70 84 93 100</td>
</tr>
<tr>
<td>Sheath</td>
<td>No effect</td>
</tr>
</tbody>
</table>
Therefore, it is considered that the *Myristica fragrans* kernel and *Syzygium aromaticum* clove stalk without bulbous head would play a key role in reducing the time that is required to obtain a 100 percent kill rate of all HAB cells. Figure 2 shows the killing rate curves of 8 species of HAB cells (*Cochlodinium polykrikoides*, *Gymnodinium catenatum*, *Dinophysis caudate*, *Prorocentrum micans*, *Pyrodinium bahamense*, *Ceratium furca*, *Karenia mikimotoi* and *Protoperidinium* spp.) in filtered seawater containing 0.3% of refined milled herbal mixtures comprises of formula one (see Fig. 1 above: 6 volumes of *Pimpinella anisum*, 3 volume of whole *Myristica fragrans* and 1 volume of whole cloves *Syzygium aromaticum*) and formula two (6 volumes of *Pimpinella anisum*, 3 volumes of *Myristica fragrans* kernel and 1 volume of *Syzygium aromaticum* cloves stalk). The number of live 8 HAB cells in the presence of *Myristica fragrans* kernel and *Syzygium aromaticum* stalk in formula two have instantly rapidly and a sharp decrease in the live HAB cells whereas over 80 percent killing rate obtained at 2 min and kill rate reached 100 percent 4 to 5 min in comparisons slower kill rate and 100 percent can be achieved after 7 min. More importantly, these results suggest that the using of *Syzygium aromaticum* clove stalk and *Myristica fragrans* kernel and *Syzygium aromaticum* increased HAB cell-killing efficiency rapidly which is important since *Cochlodinium polykrikoides* that appeared precipitated by scattering loss in open seawater and needs rapid sharp red tidecidal phyto agent capable to eliminate them before causing additional red tides.

Applying method over HAB infected area of the sea

Seawater region

At Dibba, coastal region at the northeastern tip of the UAE on the Gulf, 30 meter square (m$^2$) of seawater affected with 8 mixed species of dinoflagellates *Cochlodinium polykrikoides*, *Gymnodinium catenatum*, *Dinophysis caudate*, *Prorocentrum micans*, *Pyrodinium bahamense*, *Ceratium furca*, *Karenia mikimotoi* and *Protoperidinium* spp. was identified, marked and used for red-tide treatment. The depth of HAB, overflow water, seawater wind surface, surface
water flow, water temperature, water pH, dissolved oxygen, water density ($\sigma_\theta$) and salinity (psu) were determined.

**Experimental design**

We used ships sailing at speed 1.5m sec$^{-1}$ (Fig. 3) through the red tide infected area. Invented splashing guns are used to splash refined milled dried seeds powder mixture comprising 6 volumes of *Pimpinella anisum* (95% purity), 3 volumes of *Myristica fragrans* kernel and 1 volume of *Syzygium aromaticum* cloves stalk on the seawater surface affected with 8 mixed species of 8 mixed species of dinoflagellates in average the seawater contained approximately $>7 \times 10^4$ cells ml$^{-1}$ of the red tide in which the *Cochlodinium polykrikoides* is the most dominant species $>2 \times 10^3$ at the water surface.

Splashing of the mixed powder performs from group of holes in the front and back-end of the ship (Fig. 3). Groups of holes through hoses extended from the bottom of the ship to pump powder inside the water by depth of 0.5-1 m in the average of 150g per 1 meter square of seawater. Accordingly, water is treated during one-sail ship. The application was repeated three times with intervals of 30 min on each spot (front, depth and back).

After each treatment, samples of seawater were withdrawn at appropriate time intervals (1, 2, 3, 4, 5, 10, 15 and 30 min) and were examined under stereomicroscope to observe the HAB killing rates.

**Results and Discussion**

Figure 4 shows the killing rates of various HAB species in open seawater, HAB affected areas, after the splashing of 150g per 1 meter square of refined milled dried seeds powder mixture comprising 6 volumes of *Pimpinella anisum* (95% purity), 3 volumes of *Myristica fragrans* kernel and 1 volume of *Syzygium aromaticum* cloves stalks. The microscopic test revealed that the killing rates of HAB cells increased rapidly and reached maximum up to 62, 81 and 96, at or after 4 min during the first, second and third application, respectively (Fig. 4). Though third application has maintained the HAB killing to almost 80% after 30
min despite the seawater wind surface inference was 5.14 m sec\(^{-1}\) and water wave 0.3048, however, during the experiment the water salinity, density, were not changed, save for, the pH and percent of dissolved oxygen have reduced slightly after the treatment (Table 6). In general, the wave, flow water and wind are all collectively didn't affect the stability of the red tidecidal phyto formula in killing HAB in the treated areas. It is worth noting that it is impossible to maintain high killing percentage of HAB since the experiment was carried-out in limited area (30 m\(^2\)) from a large affected area with HAB in an open seawater as it is always expected that some live HAB will transfer from untreated water into the treated area by various mechanisms swimming with water flow, wave and wind.

**Table 6.** Average characteristics for Dibba surface water (sample depth less than 5 m) by month of March 2009. Seawater characteristics were measured before treatment and after treatment with anti-HAB.

<table>
<thead>
<tr>
<th>Parameters of seawater</th>
<th>Before first application</th>
<th>After third application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>24.6°C</td>
<td>24.6°C</td>
</tr>
<tr>
<td>Dissolved Oxygen</td>
<td>61%</td>
<td>57%</td>
</tr>
<tr>
<td>pH</td>
<td>8</td>
<td>7.9</td>
</tr>
<tr>
<td>Salinity</td>
<td>36.7 (psu)</td>
<td>36.7 (psu)</td>
</tr>
<tr>
<td>Density</td>
<td>23.9 (σθ)</td>
<td>23.9 (σθ)</td>
</tr>
<tr>
<td>Wind speed</td>
<td>5.14 m sec(^{-1})</td>
<td>5.14 m sec(^{-1})</td>
</tr>
<tr>
<td>Wave</td>
<td>0.3048 meter</td>
<td>0.3048 meter</td>
</tr>
</tbody>
</table>

After 30 min from the treatment, the red tidecidal phyto formula has swayed on the affected area creating clear HAB-negative zone on surface seawater areas whereas they were visually distinguish from that untreated areas. The red tide treated with refined milled seeds powder was seen attached with herbal powder materials suspended and floated to the surface and some found on seacoast area and some were precipitated on the rocks and stones of the seacoast. In general the dead HAB appeared can easily be removed from the seawater surface or coast.

Obviously the presence of *Pimpinella anisum* at purity of 95% in the red tidecidal phyto formula has helped to make strong attachments with HAB cells as
reported above creating an aggregate or cluster together facilitate as trapping mechanism for other live HAB. On the other hand, the presence of other refined milled powder in the formula (nucleus of *Myristica fragrans* and stem omitted bulbous head of *Syzygium aromaticum* cloves) has rapidly killed HAB cells. From the microscopic observation it seems to be, red tidecidal phyto material has provided a rupturing action on HAB cell wall (structure that protects and supports the cell) creating pores in which rendering the HAB of losing some of its cytoplasm materials "the jellylike substance" cause disturbance on the cell's osmotic pressure by allowing the cell (HAB) to be rapidly refilled by the surrounding diffusion of the red tidecidal phyto activities substances from the refined milled powder small particles size and the HAB cells eventually were expand rupture and lysis.

References


CLAIMS

1. A method for treating or preventing harmful algal bloom, which comprises applying to the affected or susceptible waters a composition comprising powdered dried plant material of Syzygium aromaticum and of at least one plant selected from Myristica fragrans and Pimpinella anisum.

2. A method according to claim 1, wherein the composition comprises powdered dried plant material of Syzygium aromaticum, Myristica fragrans and Pimpinella anisum.

3. A method according claim 1 or claim 2, wherein the plant material of Syzygium aromaticum is cloves.

4. A method according claim 1 or claim 2, wherein the plant material of Syzygium aromaticum is clove stalks.

5. A method according to any preceding claim, wherein the composition comprises Myristica fragrans and the plant material is nutmeg seed.

6. A method according to any preceding claim, wherein the composition comprises Myristica fragrans and the plant material is nutmeg seed kernel.

7. A method according to any preceding claim, wherein the composition comprises Pimpinella anisum and the plant material is aniseed.

8. A method according to any of claims 1 to 7, wherein the composition is applied to seawater.

9. A method according to any of claims 1 to 8, wherein the composition is delivered below the surface of the affected or susceptible waters.
10. A method according to any of claims 1 to 9, wherein the harmful algal bloom comprises *Cochlodinium polykrikoides*.

11. A method for killing *Cochlodinium* spp., which comprises treating the organism with a composition comprising powdered dried plant material of *Syzygium aromaticum* and of at least one plant selected from *Myristica fragrans* and *Pimpinella anisum*.

12. A method according to claim 11, wherein the composition comprises powdered dried plant material of *Syzygium aromaticum, Myristica fragrans* and *Pimpinella anisum*.

13. A method according to claim 11 or claim 12, wherein the *Cochlodinium* sp. is *Cochlodinium polykrikoides*.

14. A method for disinfecting vessels and other marine tools, instruments and facilities contaminated with harmful algal bloom, which comprises applying to the vessels, tools, instruments or facilities a composition comprising powdered dried plant material of *Syzygium aromaticum* and of at least one plant selected from *Myristica fragrans* and *Pimpinella anisum*.

15. A method according to claim 14, wherein the composition comprises powdered dried plant material of *Syzygium aromaticum, Myristica fragrans* and *Pimpinella anisum*.

16. An algicidal formulation comprising powdered dried plant material of *Syzygium aromaticum* and of at least one plant selected from *Myristica fragrans* and *Pimpinella anisum*, and one or more vehicles, carriers, binders or excipients.
17. An algicidal formulation according to claim 16, which comprises powdered dried plant material of *Syzygium aromaticum, Myristica fragrans* and *Pimpinella anisum*.

18. Use of an algicidal formulation according to claim 16 or claim 17 for the treatment or prevention of red tides in open seawater.

19. Use of an algicidal formulation according to claim 16 or claim 17 for the disinfection of vessels and other marine tools, instruments and facilities contaminated with harmful algal bloom.
Figure 2

Percentage of killing rate of HAB

Time (min)

Formula 1

Formula 2
Figure 4
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV.: A01N65/08 A01N65/28

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)
AOIN

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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D. Further documents are listed in the continuation of Box C

X See patent family annex

* Special categories of cited documents
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier document but published on or after the international filing date
  "L" document which may throw doubts on practicability claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A" document member of the same patent family

Date of the actual completion of the international search
14 June 2010

Date of mailing of the international search report
21/06/2010

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European Patent Office P.B. 5818 Patentaald 2 NL-2280 HN Rijswijk Tel (+31-70) 340-2040, Fax (+31-70) 340-3016

Authorized officer
Bertrand, Franck

Form: PCT/ISA/210 (second sheet) (April 2005)
### INTERNATIONAL SEARCH REPORT

Information on patent family members

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