UNIVERSITY OF GRONINGEN

How good is the scientific evidence that Harmful Algal Blooms are caused by non-native species and introduced by ballast water?

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Abstract: Harmful algal blooms (HAB) are caused by several phytoplankton species worldwide. The reports on them have increased since the 1970s. The HAB causing species can be divided into two groups, toxic and non toxic. When HABs are caused by non-native species, they first have to be introduced first into the new area, this is described by propagule pressure. After the introduction the species needs to invade successful in order to become part of the new environment. What is left after that phase is the achievement to become dominant, only then can a new introduced algae cause a bloom. Ballast water is pointed out as a potential introduction vector, but there are other mechanisms as well that induce the forming of a HAB as well, those are be utilization, eutrophication and climate conditions. In this thesis, the focus will be on the data that is collected in several case studies which could prove that ballast water is the introduction vector of non-native algae, which could theoretically cause a HAB in the new environment. Four case studies will be analysed on their used methods, results and formed conclusions. The collected data turned out to be circumstantial evidence, no convincing evident was provided to prove that ballast water was indeed the introduction vector in these four cases.

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Introduction

When algae increase themselves in high numbers, blooming, it is possible that they cause problems. This is either done by the production of toxins, forming a high biomass or by their physical shape (Gilbert et al., 2005). These blooms can be formed by native or non-native algae, the problems they cause have a negative effect on the environment and human health. The algal blooms that meet these criteria can be identified as harmful algal blooms (HAB). Smayda (2007) reviewed that HABs were reported more often since the 1970s, these involved species that were previously unrecorded in the area affected. This phenomenon is considered a "global increase in harmful algal blooms" (Smayda, 2007).

Not all algae cause a HAB, and even when they do, their effects can be different. Most HABs are caused by dinoflagellates, cyanobacteria and diatoms, and can be divided into two categories: toxic or non-toxic. The toxic HABs are not always dangerous to the human health, some only harm the fish and invertebrates, Hallegraeff (1993) listed a portion of the HAB causing species (Table 1).

The non-toxic blooms can have their harmful effects in two manners, by their high volume or their physical shape. The first manner, the high volume, causes harm due to increasing respiration. The effect of this increased respiration is anoxia; this will lead to the death of fish and invertebrates. The anoxia can also be a side effect of a bloom, namely by the respiration of the bacteria that occur when the decay of a HAB sets in (Hallegraeff, 1993; Campbell, 2011). The death of fish or invertebrates does not only effect the environment they are in, it can also cause negative economic effects, especially due to losses in aquaculture and fisheries. Another effect that is induced by the high volume is deoxygenation. The second manner these non-toxic blooms can cause harm is due to their physical shape, they can be either hard to eat or clog the fish gills (Campbell, 2011).

Toxic HABs can either cause harm to both humans, fish and invertebrates, or only to fish and invertebrates. The toxins cause different poisonous affects: Paralytic Shellfish Poisoning (PSP) by saxitoxins, Diarrhetic Shellfish Poisoning (DSP) by okadaic acid, Amnesic Shellfish Poisoning (ASP) by domoic acid, Ciguatera Fishfood Poisoning, Neurotoxic Shellfish Poisoning (NSP) by brevetoxins and Cyanobacterial Toxin Poisoning (CTP). Species that produce these toxins, reach humans through the food chain, by eating the fish and bivalves that have consumed these toxic species, the illnesses that these toxic species cause varies from a headache to death by respiratory failure (Hallegraeff, 1993). Eventually a HAB could also lead to the extinction of local species, by changes brought to the existing food web (Scholin et al., 2000).

If HABs are caused by non-indigenous species, they first have to be introduced into a new area. The potential of an introduction can be described by propagule pressure, which stands for: "A measure of the number of individuals released into an area which they are not indigenous" (Johnston et al., 2009). It is very well possible that the introduced propagule, for instance cysts, never become part of the population at the receiving location (Johnston et al., 2009), only a small part of the introduced propagule actually knows how to establish itself. The higher the propagule pressure gets, meaning that more propagules arrive at an area, the bigger the potential invasive success. A vector that could increase the pressure would be ballast water. The genetic characteristics of a species in combination with the harsh conditions during the transport (darkness, anoxia, fluctuating salinity levels, temperature and nutrient availability (Villac and Kaczmarska, 2011)) determine whether a species can survive (Johnston et al., 2009).

A successful introduction by propagule pressure does not stand for a successful invasion, in order to achieve that a species has to be able to adapt to a wide range of environmental conditions and factors such as competitors and grazers, these can be revered to as biotic and abiotic filters (Johnston et al., 2009). Smayda (2002, 2007) described a model, existing out of three stages, that could explain how a non-indigenous species can successfully invade, or colonize as you might say, in a new environment. Stage **I** Pioneering, stage **II** Persistence and stage **III** Community entry. In stage I, it is not the total amount of introduced cysts (propagule) that leads to a successful colonization, what is important are the genes that are present in the introduced and surviving cysts population (Smayda, 2002).These genes should be able to adapt to the physical conditions at the receiving location.

Table 1
Species that cause harmful algal blooms (Hallegraeff, 1993).

Non-toxic HABs
Dinoflagellates:
Gonyaulax polygramma, Noctiluca scintillans, Scrippsiella trochoidea, Aureococcus anophagefferens
Cyanobacterium:
Trichodesmium erythraeum
Diatom:
Chaetoceros convolutus
Toxic HABs
Paralytic Shellfish Poisoning (PSP)
Dinoflagellates:
Alexandrium acatenella, A. catenella, A. cohorticula, A. Jundyense, A. Jraterculus, A. minutum, A. tamarense, Gymnodinium catenatum, Pyrodinium bahamense
Diarrhetic Shellfish Poisoning (DSP)
Dinoflagellates:
Dinophysis acuta, D. acuminata, D.fortii, D. norvegica, D. mitra, D. rotundata, Prorocentrum lima
Amnesic Shellfish Poisoning (ASP)
Diatoms:
Nitzschia pungens, N. pseudodelicatissima, N. pseudoseriata
Ciguatera Fishfood Poisoning
Dinoflagellates:
Gambierdiscus toxicus, Ostreopsis spp., Prorocentrum spp.
Neurotoxic Shellfish Poisoning (NSP)
Dinoflagellate
Gymnodinium breve
Cyanobacterial Toxin Poisoning
Cyanobacteria:
Anabaena jlos-aquae, Microcystis aeruginosa, Nodularia spumigena

Not toxic to humans, only to fish and invertebrates
Dinoflagellate:
Gymnodinium mikimotoi

The success rate of emigrant species to colonize could increase when the same species would be introduced from different donor locations to one receiving location (Smayda, 2002). Stage II, the cysts that survived the first stage enter stage II, these surviving cysts are most likely to form a small group. Their main goal in this stage is to increase both the abundance and the genetic diversity in the second generation of the population (Smayda, 2002).When the population survived the second stage it will enter the last stage of the colonization. Stage III, when a new species is introduced in a new ecosystem and tries to establish itself, it needs to interact with the species that are already present in this environment. The species has to find a place in the existing structure of these resident species, there are two ways to achieve that; by outcompeting (some) of the resident species or to occupy a niche that has not been taken yet. After a species has survived phase III, it is part of the community it was introduced to (Smayda, 2002).Completion of the three described stages only provides the new species a position in the community, it is not a given that this emigrant species will cause a HAB. In order to reach that phase it has to become a dominant species in the community, when it does not become dominant it will likely become part of the hidden flora (Smayda, 2002).

Ballast water was mentioned as a vector that could increase propagule pressure, because of the increasing amounts of ballast water and the high transportation frequency (Smayda, 2007). The actual function of ballast water can be described as: “Water placed in a ship to increase the draft, change the trim, regulate the stability, or to maintain stress loads within acceptable limits; it includes the sediment that accumulates in ballast tanks and holds” (Steichen et al., 2012). The water used as ballast water is taken from coastal waters, it is placed in ships at the start of their journey and very often dumped at the end of this journey. Bolch and de Salas (2007) and Boltovskoy et al. (2011) mention that the attention for ballast water, concerning the introduction of non-native species, started in the 1970s. During that period the examination of ballast water was only focused on vertebrates and invertebrates, it was not until the beginning of the 1990s, according to Bolch and de Salas (2007) that the ballast water inspections also focused on the transport of phytoplankton. Until then, it was not known that the ballast water still contained a high level of living phytoplankton at the time of their arrival at the receiving ports. After the discovery of viable toxic microalgae as part of the viable phytoplankton after a journey, ballast water was suspected to be a major vector for the global transportation of these toxic microalgae (Bolch and de Salas, 2007).

Besides the increasing propagule pressure by ballast water, there are other aspects that influence a potential HAB. There are three other mechanisms that play a part in this phenomenon: increased utilization of coastal waters, stimulation of plankton blooms by cultural eutrophication and changing climatological conditions (Hallegraeff, 1993; Smayda, 2002). And the physical conditions play a part as well. The increased utilization of coastal waters, due to the increasing aquaculture industry a more sensitive ecosystem is created, which can trigger the species that create HABs either native or non-native species (Hallegraeff, 1993; Smayda, 2002). Because of cultural eutrophication, a change is brought tothe living situation, which can also stimulate HABs. By adding extra nutrients (for instance Nitrate and Phosphate) to the coastal waters, species that would normally not survive or bloom at that specific location, now find themselves in a better suited living situation (Hallegraeff, 1993; Smayda, 2002; Smayda, 2007). The last mechanism that plays a role in the HAB occurrence is the changing climatological condition; this can also be indicated as a factor that changes the living environment. These changing conditions include global warming and El Niño events (Hallegraeff, 1993; Smayda, 2002; Smayda, 2007). Another aspect plays part in the reports of HABs, is the increased scientific awareness, ever since the better understanding of the negative impacts that HABs have, more research has been done on them (Hallegraeff, 1993). The increased scientific awareness does not contribute to the occurrence of HABs, it increased the attention and reports of them.

There are biotic and abiotic factors that are involved in HABs, the biotic factors being the competition and grazers present at a new receiving site. The abiotic factors are temperature, salinity level, available nutrients and the light intensity. All the previous described factor that influence the occurrence of a HAB are displayed in Fig.1.

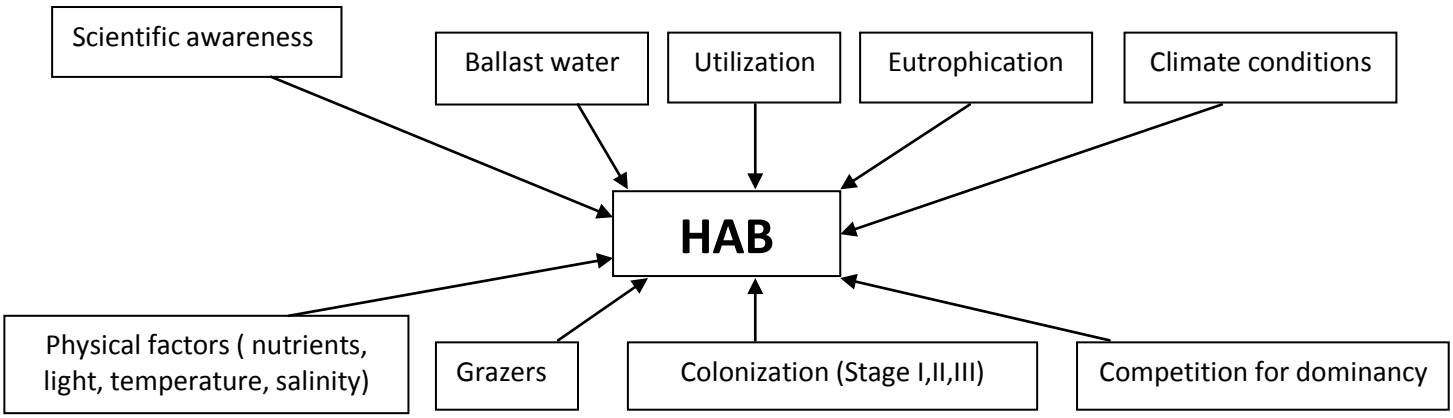

Fig.1. A model that presents the complexity of the existence of a HAB. There are multiple factors that influence a specie before it can actually bloom.

The two hypotheses in this thesis are: A HAB is caused by native species, they are triggered by factors such as increasing eutrophication or changing climate conditions. The second hypothesis is: A HAB is caused by non-native species, they were introduced into the new area and after a successful invasion became dominant.

The aim of this thesis is to review the given evidence that HABs are caused by ballast water and that the species causing these HABs are actually non-native to the area.

Material and methods

In this thesis, literature was reviewed, the search for relevant articles started on the Web of Science. The search criteria was wide at the beginning, words as ballast water/ harmful algal blooms/invasive species gave a lot of hits, sometimes more than a thousand. Out of these numerous hits, the articles were selected on their title, abstract or keywords. These first articles gave information in general on ballast water and HABs. When the search was refined, by focussing on a few HAB outbreaks and the research done on them, the outline of the thesis was formed. The case studies were selected in a random manner. At that point I had read approximately forty articles. In the search, no attention was paid to the papers that published the articles and how many times the article was cited. The year that the article was published was sometime of importance, my preference went out to the articles with the most recent data when multiple articles covered the same subject.

The articles on HAB outbreaks use different methods to determine whether a HAB causing species is native or not and how it was introduced. Bolch and de Salas (2007) and Smayda (2007) formed a list of the research methods that can be used to determine from where and if a HAB causing species was introduced. Research methods: Historical distribution records, sediment dating studies, analyses on living material (toxicity profiles/ mating studies/molecular data) and Bio geographical data. The historical distribution records provide information about global shipping routes, knowing these routes and the donor locations can add additional information to theories on ballast water as an introducing factor. Sediment dating studies can be used when the HAB causing species have a cyst phase, these cysts are stored in the sediment. Via sediment core studies it is possible to determine the age of a species at the sampled location. By analysing the living material, multiple stages are included to give information on the species itself. The toxic profiles provide information on the exact morphospecies, the mating studies are done between strains of different populations, this provides information on the relatedness between assumed donor populations and the invasive populations. The last possible research done on the living material is the analysis of the molecular data, to compare DNA of the algae strain at the bloom location to algae of the possible donor strain, this will provide information on the relation between those strains. By collecting bio geographical data, a researcher can gain information on the distribution of species. This list of research methods will be revered to as the recommended methods.

The used methods in the five case studies that were reviewed were analysed and then compared to the recommended research methods. The four HAB outbreaks used are: *Alexandrium catenella,* dinoflagellate causing PSP at Thau Lagoon (France); *Gymnodinium catenatum,* dinoflagellate causing PSP at Tasmania (Australia), New Zealand and the Iberian Peninsula (Portugal, Spain)

Results

Used methods and results of case studies

Alexandrium catenella, at Thau Lagoon (France)

Prior to the bloom of *Alexandrium catenella* at Thau Lagoon in 1998, a long term phytoplankton monitoring programme had been running for over a decade, with at least two surveys a month along the coast. In the year 1995 *A. catenella* was first discovered. During the first bloom, water samples were taken and the following methods were used to determine the origin of this species: Toxin composition, restriction fragment length polymorphism (RFLP) and DNA sequence comparisons, these researches were done by Lilly et al. (2002).

The cultures derived from the water samples were first used to identify the actual species, it was identified as A*. catenella.* The RFLP pattern was compared to several other strains (Japan, Italy, Scotland and England), the pattern was identical to the Japanese Temperate Asian ribotype (Fig. 2) The other strains differed from the Thau Lagoon pattern.

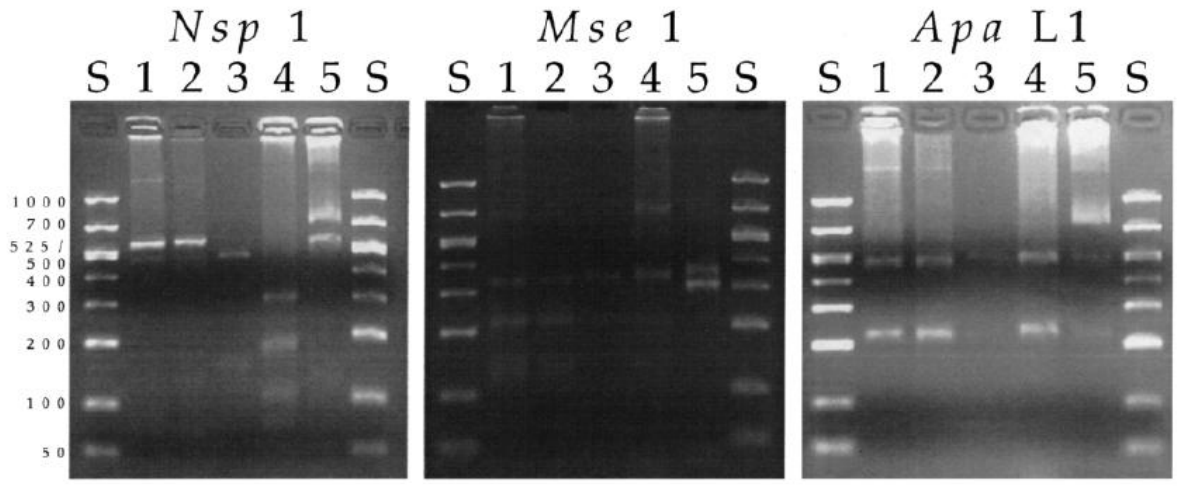

Fig. 2. (Lilly et al., 2002) The RFLP patterns of the *Alexandrium catenella* strains. Fragments were generated with the enzyms Nsp 1, Mse 1 and Apa L1. Lane S, molecular weight standards; lane 1, Thau Lagoon, lane 2, Japan; lane 3, Italy; lane 4, Scotland; lane 5, England.

The DNA sequence of LSU and rDNA were compared with sequences of other *A. catenella* species and strains that Scholin et al. (1994) had published. The Thau Lagoon strain showed the most resemblance with the Japanese Temperate Asian ribotype, of the 709 bp, only two of them differed.

The toxin analysis confirmed that the Thau Lagoon strain was toxic. Of the two cultures that were derived from the water samples taken of the bloom, the toxin content (expressed in fg saxitoxin (STX) equivalents/cell) was 44.3 fg STX equivalents/cell and 5.3 fg STX equivalents/cell. The Japanese strain had a toxin contend of 22.6 fg STX equivalents/cell and the Scottish strain a toxin content of 127.4 fg STX equivalents/cell. The strains from Italy and England did not contain toxins.

Gymnodinium catenatum, at Tasmania (Australia)

The first registered bloom of *Gymnodinium catenatum* was in 1985, at Derwent Estuary (Hobart, Tasmania). Since then several studies have been done to determine the potential donor location of this species and the year it was presumably introduced. The following methods were applied: Sediment dating studies, DNA sequencing and mating studies. The cultures that were used for these

methods came from plankton net samples or cysts derived from sediment samples. The samples were either collected at Australian or overseas locations.

The sediment dating study was carried out by McMinn et al. (1997). Sediment cores were collected in two different years at Deep bay in southern Tasmania. In the year 1991, the cores were 15 to 25 cm long and 4.5 cm in diameter and in 1994 they were 80 cm long and 11 in cm diameter. These cores were examined on the presence of *G. catenatum* cysts (Table 2) and in combination with the Pb 210 method, the age of the sediment consisting the cysts was determined (Fig.3). The 210Pd method in combination with the cyst analysis revealed that there were no cysts found that were older than 1972 ±2 year in the 1991 cores or older than 1937 ± 6 year in the 1994 cores.

Table 2. (McMinn et al., 1997)
Distribution of dinoflagellate cysts with depth in the 1991 and 1994 sediment cores from Deep Ray, Tasmania. All abundances are given as percentage abundance. Names in parentheses are palaeontological names.

Species	1991 core													
Depth (cm):	1	2	3	4	5	6	7	8	9	10	11	12	14	15
Gymnodinium catenatum	0.4	1.3	0.6	0.3	0.8	0.2	0.3	0.3	0.3	0	0	0	0	0
Protoceratium reticulatum	90.7	93.0	92.8	92.4	89.0	93.0	91.7	91.3	93.2	94.5	93.0	93.7	93.5	89.5
Polykrikos schwartzii	0.2	0	0	0.1	0.9	0.1	0.7	0.1	0.1	0.1	0.2	0.2	0	0
Protoperidinium spp.	0.7	0.4	0.2	0.7	1.2	0.5	0.2	0.9	0.1	0	0	0.2	0	0
Gonyaulax scrippsae	0.9	1.4	1.9	1.8	1.3	0.5	0.7	0.5	0.4	0.5	1.6	0.7	1.4	4.5
Gonyaulax spinifera (= *S. mirabilis*)	5.4	4.9	4.6	4.6	4.9	4.7	6.0	5.7	6.0	4.7	4.9	3.7	4.7	5.4
Gonyaulax spinifera (= *S. ramosus*)	0.2	0	0	0	1.1	0.5	0.2	0.7	0.2	0.2	0.2	0.98	0	0.2
Gonyaulax spinifera (= *S. membranaceus*)	1.4	0.1	0.2	0.2	0.2	0	0.2	0.7	0	0	0	0.5	0.5	0.7

	1994 core													
Depth (cm):	1	2	3	7	9	11	13	15	17	19	21	23	25	27
Gymnodinium catenatum	0.8	0.8	1.6	0.9	0.9	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Protoceratium reticulatum	93.0	93.7	93.7	91.6	94.3	95.8	92.2	95.8	95.7	96.3	95.4	95.8	95.4	96.1
Polykrikos schwartzii	0.2	0.2	0.3	0.1	0.2	0.2	0.1	0.2	0.1	0.3	0.1	0.2	0.2	0.3
Protoperidinium spp.	0.4	0.6	0.6	0.3	0.3	0.2	0.4	0.4	0.3	0.2	0.2	0.2	0.3	0.3
Gonyaulax scrippsae	0.5	0.6	0.8	0.5	0.8	0.6	0.3	0.2	0.3	0.6	0.1	0.1	0.0	0.2
Gonyaulax spinifera (= *S. mirabilis*)	3.2	2.8	0.3	5.2	3.2	2.4	6.4	3.3	3.5	2.7	3.8	3.5	4.1	3.1
Gonyaulax spinifera (= *S. ramosus*)	1.8	1.2	0.8	1.2	0.3	0.4	0.4	0.0	0.0	0.1	0.3	0.1	0.0	0.0
Gonyaulax spinifera (= *S. membranaceus*)	0.2	0.1	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0

Depth, continued (cm):	29	31	33	35	37	39	45	51	55	60	65	70	75	80
Gymnodinium catenatum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Protoceratium reticulatum	96.0	96.4	94.8	95.0	94.0	96.2	95.7	96.3	96.2	9.6	95.6	96.0	95.8	97.1
Polykrikos schwartzii	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.1	0.0	0.1	0.2
Protoperidinium spp.	0.5	0.2	0.1	0.2	0.5	0.3	0.3	0.3	0.3	0.4	0.3	0.2	0.3	0.3
Gonyaulax scrippsae	0.2	0.2	0.3	0.2	0.3	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.0	0.0
Gonyaulax spinifera (= *S. mirabilis*)	2.8	2.7	4.2	3.8	4.2	2.7	3.1	2.7	2.9	2.9	3.7	3.3	3.2	0.3
Gonyaulax spinifera (= *S. ramosus*)	0.2	0.2	0.4	0.5	0.7	0.3	0.3	0.2	0.4	0.3	0.2	0.2	0.4	1.9
Gonyaulax spinifera (= *S. membranaceus*)	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.2

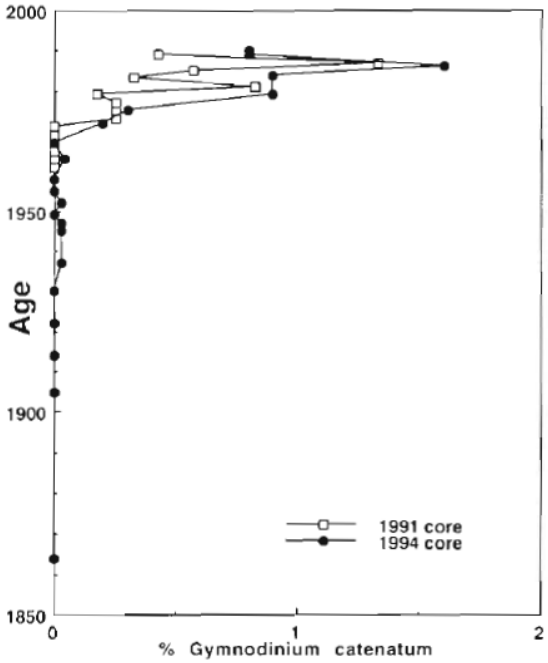

Fig. 3. (McMinn et al., 1997) *Gymnodinium calenatum* Abundance of cysts (expressed as percentage of total dinoflagellate cysts) with date for 2 sediment cores from Deep Bay, Tasmania.

Table 3 (Bolch and Salas, 2007)
Summary of 63 *Gymnodinium catenatum* strains for which the 5.8SrDNA C/T polymorphism has been determined.

Population	Strains *N*	Year isolated	5.8S rDNA SNP
Australia	34		
Tasmania	27	1986–1993	T
Port Lincoln, SA	3	1996–1998	T
Port Phillip Bay, Vic.[a]	1	1993	T
Cowans Creek, NSW	3	1997	T
New Zealand	2	2000	T
Europe	9		
Spain	6	1985	C
Portugal	3	1989	C
South America	4		
Uruguay	4	1998	C
South-east Asia	14		
Singapore	2	1999	C
Hong Kong	4	1998	C
Korea	4	1998–1999	C
Japan	3	1993–1995	C
GCJP01	1	1985	T

[a] DNA extract from strain *G. catenatum* MUCC, isolated from Port Phillip Bay, 1993.

The rDNA-ITS sequencing, performed by Bolch and Salas (2007) was done on 63 (Table3) strains. There turned out to be a single nucleotide polymorphism (SNP) on the 5th base of the 5.8SrRNA gene. This SNP led to two different rDNA-ITS ribotypes, the C-gene strain and the T-gene strain. The C-gene strain carried cytosine on the 5th base of the 5.8SrRNA gene, and the T-gene strain carried thymidine on that same location. The T-gene was only found at the Australian, New Zealand and Japanese (Seto Inland Sea) strains (Table3).

The mating study was done with strains from Spain, Japan and Australia, the results in Table 4 were derived from Bolch et al. (1999a,b) and Blackburn et al. (2001). The outcome was a high viability between the strains of Spain and Japan (80%), and a low viability between Australia and Spain (5%) and Japan (10%). Suggesting that the Australian strain does not have the same origin as the Spanish and Japanese strains used in this study.

Table 4 (Bolch and Salas, 2007) Summary of RAPD similarity and mating compatibility between the Australian, Japanese and Spanish strains of *Gymnodinium catenatum*

Pairwise comparison	Pop^n RAPD similarity[a]	Mating compatibility[b]	Post meiotic viability (%)[b]
Japan–Spain	0.757	2.25	80
Australia–Spain	0.713	1.54	5
Australia–Japan	0.702	0.60	10

[a] Bolch et al. (1999a,b).
[b] Blackburn et al. (2001).

Gymnodinium catenatum, at New Zealand

The first reported bloom at the west coast of the New Zealand Northern Island was in 2000. There was a biotoxin monitoring program since 1993 to detect harmful algae, which measured water samples at approximately seventy sites (Irwin et al., 2003), the algae *Gymnodinium catenatum* was not detected in the samples until it actually bloomed(Mackenzie and Beauchamp, 2002; Irwin et al., 2003). The research methods applied after this bloom were sediment dating studies and DNA sequencing.

The sediment dating study was carried out by Irwin et al. (2003), core samples were taken at three different locations in 2001, the locations along the coast of the Northern Island were: Manukau Harbour, where the bloom was first reported; Hokianga Harbour, where *G. catenatum* caused the highest shellfish toxicity and Wellington Harbour, as a control site. The cores were taken with 40 cm long tubes that were 4.5 cm in diameter. The cores were sliced and examined on the presence of *G.*

catenatum cysts. To determine the age of the sediment the cysts were found in, 210Pd radiometric dating was used. By combining these two methods, the age of the cysts was determined (Fig.4,5), the cores of Wellington Harbour could not be analysed correctly because the surface sediment mixed. The cysts of *G. catenatum* were present in Manukau Harbour since 1980 and since 1981 in Hokianga Harbour, the data collected at Wellington Harbour could not be used.

DNA sequencing was carried out by Bolch and Salas (2007), and was performed on 63 strains (Table3). A SNP was found on the 5th base of the 5.8S rRNA gene, dividing the strains into two groups. The T-gene group, carrying thymidine and the C-gene group, carrying cytosine. The T-gene was only found in the Australian and Seto Inland Sea (Japan) strains.

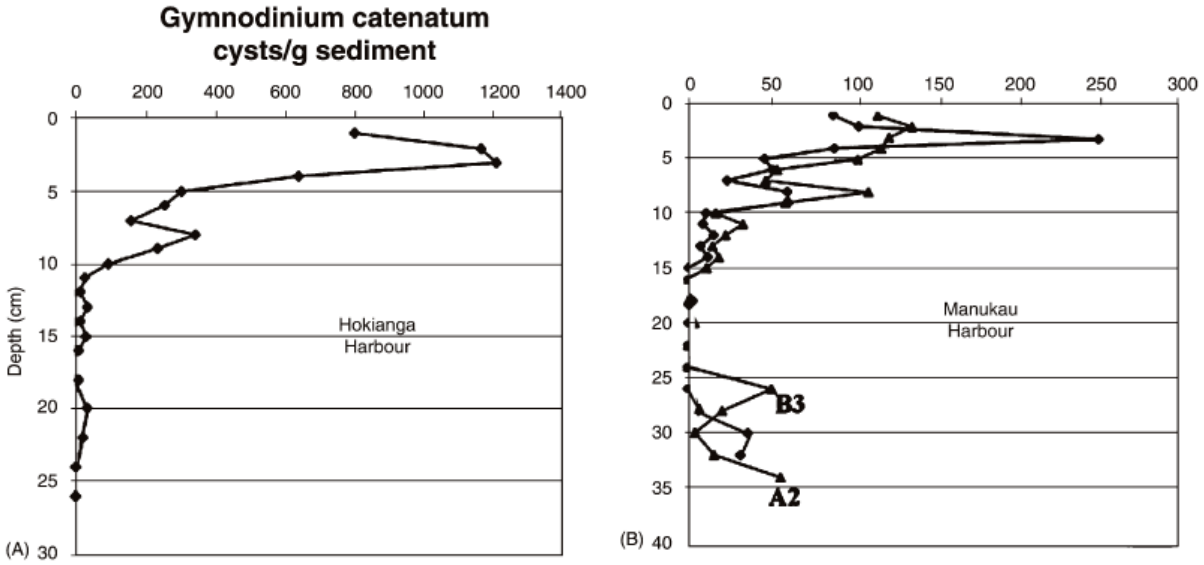

Fig. 4 (Irwin et al., 2003) Sediment data that indicates the presence of *G. catenatum* at Hokianga Harbour (A), Manukau Harbour (B). On the x-as the cysts per gram sediment, on the y-as the depth of the core.

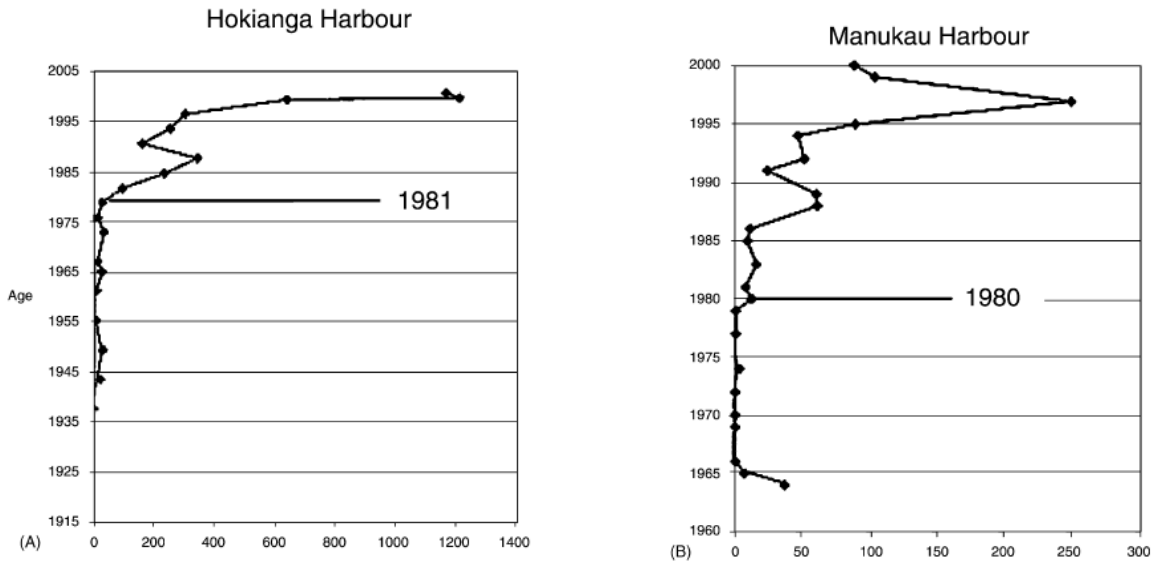

Fig.5 (Irwin et al., 2003) The year from where on the cysts started to increase in abundance. Hokianga Harbour (A), Manukau Harbour (B). On the x-as the cysts per gram sediment, on the y-as the age in years.

Gymnodinium catenatum at the Iberian Peninsula (Portugal, Spain)

Toxic blooms of *Gymnodinium catenatum* were first reported in the mid-1970s on the West coast of the Iberian Peninsula (Portugal, Spain), the reconstruction of this possible invasion was done by sediment dating studies.

The sediment dating study was carried out by Ribeiro et al. (2012), the cores were taken from three different locations along the Iberian Peninsula: Mira, Lis and Douro (Fig. 6). Sediment cores were collected in 2002 with the octopus 50x50 cm box core. On board, PVC tubes were used to collect the so called sub cores. The cores had the following lengths per location: 24 cm long at Mira, 33 cm long at Lis and 18 cm long for Douro. The age of the sediment in where the cysts were found was determined by the 210 Pd activity measurements. The data was not used when surface mixing was found.

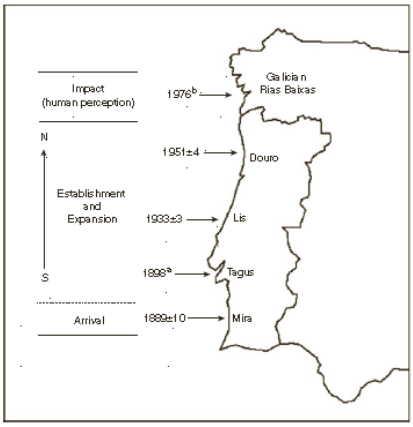

Fig. 6 (Ribeiro et al., 2012) The locations where the sediment cores were taken and the years they were introduced.

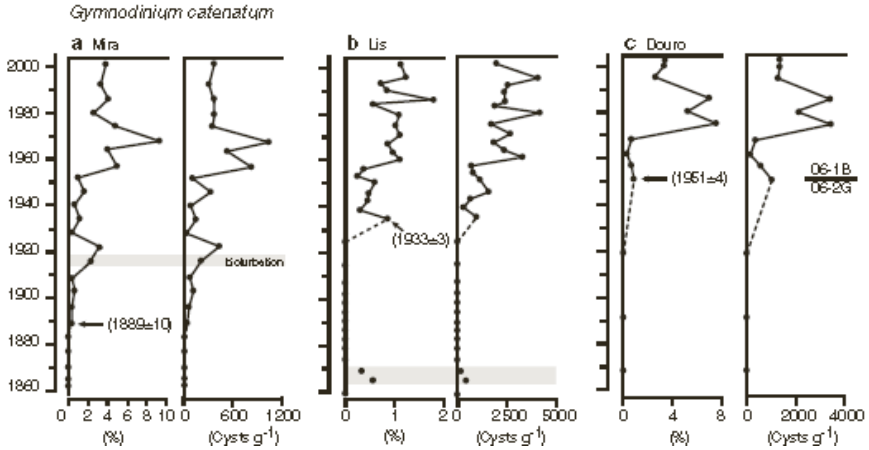

Fig. 7 (Ribeiro et al., 2012) Cyst record of *G. catenatum.* The relative abundance and cyst concentrations are presented for Mira, Lis and Douro. At the y-as the age in years, on the x-as the cysts per gram and percentage.

The data received from the sediment cores in combination with the 210 Pd data indicated that the cysts at the coast of Mira were from the year 1889 ± 10, Lis 1933 ± 3 and Douro 1951 ± 4 (Fig.7). Theses ages are based on 24 sediment samples for Mira, 33 for Lis and 13 for Douro. Because the core length at Douro was 18 cm and only covered 50 years, the data was complemented with samples of a 562 cm-long gravity core collected at the same site. Dale et al. (2005) took sediment samples at Tagus prodelta (Fig.6), located near the port of Lisbon, here *G. catenatum* first occurred around 1898.

Research methods vs. recommended methods

The material and methods section included the recommended research methods described by Bolch and de Salas (2007) and Smayda (2007), the actual used methods in the four case studies were described as were their results and conclusions. The use of the recommended methods would according to Bolch and de Salas (2007) and Smayda (2007) improve the scientific evidence to connect ballast water as a introduction vector to a HAB. Table 5 provides an overview of the used methods in each case study. None of the studies uses all the recommended research methods, most of them apply the sediment dating studies, it gives information on the year a species appeared into a region but it cannot be used for every species. The historical distribution studies are also well applied, this is a very understandable method to use. When there is no data available on the shipping routes, it is difficult to connect the donor and receiving location to each other, at least when ballast water would be the connecting factor

Table 5 An overview of the used researched methods, conclusion and other characteristics for the research area. The researched methods are the following: MD= Molecular data, TP= Toxin profiles, SDS= Sediment dating studies, BD= Biogeographical data, MS= Mating studies, HDR= Historical distribution records.

HAB location	France	Portugal, Spain	Tasmania	New Zealand
Species	*A. catenella*	*G. catenatum*	*G. catenatum*	*G. catenatum*
Data type	MD, TP, HDR	HDR, SDS, BD	MD,SDS, HDR, BD,MS	SDS, HDR, BD, MD
Sampling area	Thau Lagoon water samples	Sediment cores Iberian Peninsula	Australian plankton net samples/ Sediment cysts samples	Sediment core samples
Monitoring	Since at least 1985	-	-	Since 1993
Conclusion	Human introduction, from Asia	Natural introduction, from Africa	Human introduction, from South japan	Human introduction, from Australia or Japan

Discussion

When research is done to a species that causes a HAB, often the search is for data that could proving evidence that the HABs are caused by introduced species. The focus is on the when and where. *When* was it introduced? From *where* was it introduced? The introduction by ballast water however is not a bloom stimulation factor on itself.

The obtained results in the case studies were discussed by the authors, which led to their conclusion. Each conclusion made in the case studies, is individual discussed.

Alexandrium catenella at Thau Lagoon (France)

The conclusion by Lilly et al. (2002) was, that an human introduction of *Alexandrium catenella* in Thau Lagoon caused its presence. The arguments that were given: there was a long-term phytoplankton monitoring program, with no results for the presence of *A. catenella* until 1995; LSU and rDNA sequences and the RFLP patterns both showed (almost) identical resemblance with the Japanese strain and the toxic composition was more linked to the Japanese strain than to the Scottish strain. According to Lilly et al. (2002), the data collected was enough evidence to suggest that: “The presence of *Alexandrium catenella* in Thau lagoon is originally from the western Pacific and was introduced to the Mediterranean by ballast water transport and discharge”. They do admit however that there is still a possibility that this species was not introduced by ballast water but due to a natural cause, but according to the authors that it is not very likely. An argument that is used to back up the ballast water theory is the port of Sète close by, which is connected with Thau Lagoon (Abadie et al., 1999).

What the research of Lilly et al. (2002) has shown is that there is a genetic similarity between the *A. catenella* strains of Thau Lagoon and Japan. The strain was only compared to three other strains; therefore it is not to say that there is no other strain at a different location that is genetically similar. Concluding that this strain is introduced out of Japanese populations is too soon. Furthermore, the absence of *A. catenella* in the monitoring study does not proof that it was not present, it was not sampled, which does not proof absence. The final argument on the port of Sète, connected with Thau Lagoon, does not proof that *A. catenella* was introduced via ballast water. The

conclusion that this was a non-native species from Japan via ballast water as introduction vector is based on circumstantial evidence.

Gymnodinium catenatum at Tasmania (Australia)

Bolch and Salas (2007) conclude in their review that there is a potential recent link between the Australian strain of *Gymnodinium catenatum* and the strain from the Seto Inland Sea (Japan) and that the species was introduced by ballast water. The arguments used are: There were no reported blooms of this species prior 1985, therefore it is non-native; Historical plankton samples did not show presence of *G. catenatum* before 1980 at south Tasmania (Hallegraeff et al., 1989); The presence of the T-gene at both the Australian strain as the Japanese strain indicates the genetic relation; Sediment data revealed that *G. catenatum* occurred not earlier than 1973 (McMinn et al., 1997); No oral history known of shellfish toxicity by the Aboriginal culture and based on global distribution records of *G. catenatum* and historical shipping patterns, which fit the presumed introduction from Japan in the early 1970s.According to the sediment dating studies *G. catenatum* arrived at south Tasmania in the early 1970s. That answered the question of when it was presumably introduced, to determine the possible donor location, molecular data was collected. The DNA sequence data provided a strong link between the Australian and Seto Inland Sea strain, because of the presence of the T-gene in both these strains. This same gene could also be the reason that the mating study showed low viability (Table 4) between the Japanese and Australian strain, if a Japanese strain with the C-gene was used in this study it would indeed lead to a low viability. Assuming that *G. catenatum* was introduced in the early 1970s from Japan, there is historical shipping data that could back this up, Japanese woodchip carriers came to Tasmania in the late 1960s and early 1970s.

The T-gene strain from Seto Inland Sea has not been found there since 1985, since then only C-gene strains have been found. C-gene strain have maybe out competed the T-gene strain, or perhaps the T-gene strain was actually not native to that area but introduced itself. Because of the absence since 1985, and the year *G. catenatum* was presumably introduced, there could be a different donor location. All the arguments given, lead to believe that this species was indeed introduced, but there is an inconsistency in the sediment data. The 1991 cores show that the found cysts are not older than 1972 ±2 year and de cysts found in the 1994 cores are from the year 1937 ± 6. The core used in 1994 was 80 cm long and provided more data on the sediment history. Data from this year does not fit the ballast water theory of the introduction by woodchip carriers from Japan in the late 1960s and early 1970s. Basically what is proven, is that the strains present at Tasmania are genetically the same as a strain found at Seto Inland Sea, that was last found in 1985. And that there is some inconsistency in the sediment data, there was however an expansion in the early 1970s of cysts present in both sediment dating studies. There is no actual prove that this species was indeed introduced, because there was no natural baseline, and if introduced, there is no prove that this was done by ballast water. All the evidence is circumstantial. What should be mentioned as well, during the first bloom in 1985, shellfish industry took place at Tasmania (Bolch, 1999a), which means an increase of utilization and eutrophication. Both these mechanism can influence a HAB, as described in the.

Gymnodinium catenatum at New Zealand.

Irwin et al. (2003) concluded that *G. catenatum* was introduced by human cause, based on the sediment data and the DNA sequence. Like the Tasmanian strain, the New Zealand strain does also have the T-gene. In that sense they are connected to each other. The abundance of the cysts in the sediment grew in the early 1980s, considering that the sediment data of Tasmania shows an increase in the beginning of the 1970s, there could be a connection between these two locations. Or there is no link and the strains were introduced separately from the same donor location. In this study, it is either Tasmania or the Seto inland sea that is indicated as the donor location. According to historical

distribution studies, there has been shipping between the Manukau Harbour and Australia (Tasmania and South Australia) until 1991.

What Irwin et al. (2003) mention in their article is, that it is possible that *G. catenatum* has actually been in New Zealand longer, but because the used cores were rather short, 18-34 cm long, this could not have been determined. And when looking at Fig.4 and Fig.5, Manukau Harbour has shown a higher abundance in cysts before 1980, namely around 1964. It is therefore not to say that the introduction was actually in the 1980 and 1981. Because of the T-gene, either Tasmania or Seto Inland Sea is mentioned as possible donors. The same can be said about this assumption, as said in the Tasmanian case study. There could be another population with the T-gene as well that is not sequenced yet. And the ballast water theory is also not based on solid prove, it is all circumstantial. The Tasmanian and New Zealand case studies are similar in the sense that it concerned the same species which are genetically identical, and the same conclusions are made about their supposed introduction. What is also similar in these cases, New Zealand did also perform aqua farming of mussels, meaning utilization and eutrophication.

Gymnodinium catenatum (Portugal,Spain)

The cyst records of *G. catenatum* in the sediment indicate that the species first occurred in Mira, and from there had a northward expansion. Ribeiro et al. (2012) do not believe that *G. catenatum* was introduced by human activities into the port of Lisbon (Tagus prodelta) by ballast water; because this would not match with the results of the sediment samples. According to the authors, when it would have been introduced by ballast water at the port of Lisbon it could not been explained why the cysts found at Mira are older. The conclusion is therefore that this species was introduced in a natural manner. Taken into account in this theory is the presence of *G. catenatum* in sediment core samples taken between 20-32˚ N of the African NW coast, the cysts were between 130 and 340 years old, these sediment samples were taken by Holzwarth et al (2010a).

The conclusion that *G. catenatum* is invasive and introduced in a natural manner was solely based on sediment dating studies. And what could back up this theory is the presence of the species at the African NW coast. However, there were no genetic methods used in this study, making it impossible to connect the found species at the Iberian Peninsula to any other populations. Interestingly enough the ballast water theory was dismissed after the data was analysed.

What is well represented in Table 5 is that none of the case studies have used all the methods. There is a certain gradation in these recommended methods, not all methods are of the same importance. The molecular data and toxin profiles provide strong evidence on the relation between different strains or populations of the same species. The sediment data provides information on the year a species is presumably introduced, although theses sediment cores can give contradicting information about the age of the first cysts. The cores that were taken during those studies differ in lengths among each other; longer cores could maybe give different results. Mating studies could give useful information on the viability of mating crossings; a high viability indicates a higher relation between strains or populations. Mating can be very complex matter, and therefore will not always succeed that well. The historical distribution records are useful to make the ballast water theory more plausible, but it actually does not prove anything. That there was a shipping route between a HAB location and the presumed donor location does not prove anything yet, releasing ballast water at a port does not mean a immediate introduction. The research in Tasmania covered all but one. But having read about the methods, results and conclusions in the Tasmania case study, they still do not have the convincing evidence that *G. catenatum* is actually introduced by ballast water.

Conclusion

All four case studies base their conclusions on circumstantial evidence, none of them can really proof that the HAB causing species was non-native, and if so introduced by ballast water. Even when using almost all the recommended research methods, there is still no convincing evidence. This suggests that the recommended research methods do not provide enough data to make a convincing case. A natural baseline is very important, to know what is actually native and non-native prior to a HAB.

Furthermore, the research focussed too much on proving that ballast water is actually an introduction vector, wrong conclusions are made based on circumstantial evidence. The collected data is used as indirect evidence for ballast water as the introduction vector. The assumption is already there and the evidence is sought in that direction. Ballast water is not a HAB causing factor on its own, there are lot of factors that influence the cause of a HAB.

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