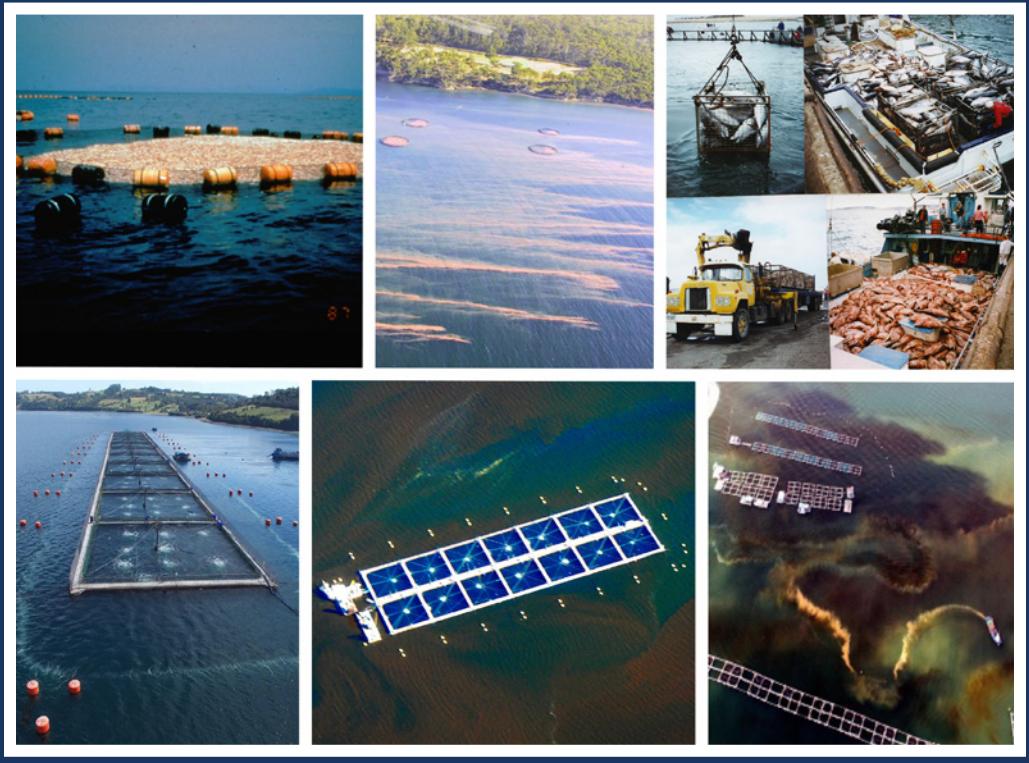
FISH-KILLING MARINE ALGAL BLOOMS: Causative Organisms, Ichthyotoxic Mechanisms, Impacts and Mitigation

G.M. Hallegraeff, D.M. Anderson, K. Davidson, F. Gianella, P.J. Hansen, H. Hegaret, M. Iwataki, T.O. Larsen, J. Mardones, L. MacKenzie, J.E. Rensel

Contributors: A.D. Cembella, O. Espinosa, L. Guzman, B. Krock, P.T. Lim, A.R. Place



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Top, from left to right: Yellowtail aquaculture mortality in the Seto Inland Sea, Japan, 1972, associated with *Chattonella marina* algal bloom; courtesy Prof T. Okaichi; *Noctiluca scintillans* red tide slicks threatening Tasmanian salmon farms, Tasmania 2003; photo J Marshall and G Hallegraeff; Clean-up after tuna aquaculture kill in Port Lincoln, Australia, 1996, associated with *Chattonella marina*; photos B. Munday and G. Hallegraeff; Bottom, from left to right: Airlift upwelling and bubble curtains used as a mitigation strategy against the 2016 *Pseudochattonella verruculosa* bloom event in Chile, photo: INTESAL; Aerial photograph of net pens with airlift upwelling during a *Heterosigma* bloom in British Columbia; photo Cermaq Canada Ltd.; Aerial view of merry-go-round clay dispersal on south coast of Korea, 2008, to protect against *Margalefidinium polykrikoides*; photo Jong-Suk Jung.

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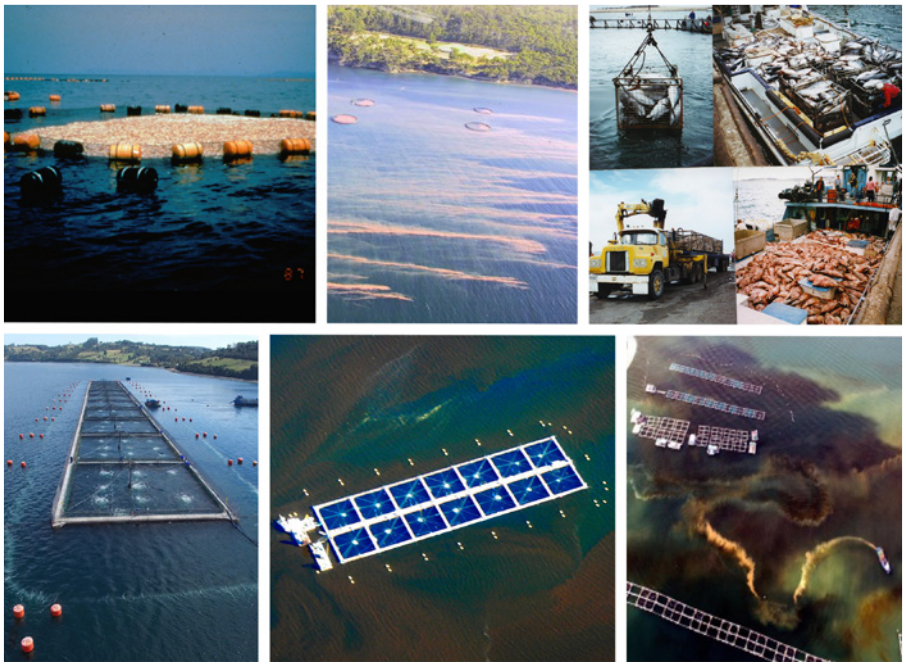

Preface and Acknowledgements

Fish-killing algal blooms are of increasing concern to socio-economic interests linked to the sustainability and security of seafood and living resources. Development of fisheries and aquaculture as part of integrated coastal resource management are particularly susceptible to the threat of ichthyotoxic events and their consequences. Whereas these events are categorized as "fish-killing", there are associated impacts on other components of coastal marine ecosystems, including wild fish populations, benthic macrofauna and macrophytes.

Outside the aquaculture and fisheries industry sector, there has been inadequate consideration of fish-killing algae and the topic has not been systematically addressed within the scientific community on a global basis. Known ichthyotoxic marine algae are usually identifiable as causative organisms, but there remain taxonomic and biogeographical uncertainties in the distribution of the species. The role of climate change leading to regime shifts and hence possible increased frequency, magnitude and biogeographical distribution of fish-killing algal blooms poses a challenge to understanding the future ocean. Knowledge of the environmental factors driving bloom dynamics are not fully understood, and this has hampered the development of predictive models for forecasting and risk assessment of fish-killing events. Even the proposed mechanisms whereby exposure to such blooms causes fish morbidity and mortalities are highly controversial and lack scientific consensus. Furthermore, there is only limited application and lack of standardization of current fish- or cell-based bioassay methods for assessing ichthyotoxicity.

In October 8-11, 2019, a GlobalHAB *Advanced International Colloquium and Technical Workshop on Fish-Killing Marine Algae and their Effects*, was held in Puerto Varas, Chile, to comprehensively address these gaps in knowledge, to provide a synthesis of current state-of-knowledge linked to strategies for technological and scientific approaches to mitigating the impacts. The present White Paper constitutes the fruitful result of the work conducted during the event.

The Workshop and this White Paper were implemented by the IOC-SCOR GlobalHAB programme. The Workshop and the publication of this White Paper was funded by the government of Chile through CORFO and the collaboration of CREAN-IFOP, and funds to GlobalHAB from UNESCO IOC Regular Programme for the IOC HAB Programme/GlobalHAB, as well as from the Scientific Committee on Oceanic Research (SCOR) with funds contributed by the national SCOR committees.

Executive Summary

Fish-killing microalgal blooms are responsible for much greater global socio-economic impacts than the well-studied HAB species causing seafood biotoxin contamination. Examples are the 1972 *Chattonella marina* bloom in the Seto Inland Sea, Japan (estimated USD 71M loss to yellowtail aquaculture), the 1988 *Prymnesium polylepis* bloom in the European Kattegat with broad marine ecosystem impacts, and the 2015/16 *Pseudochattonella verruculosa* bloom in Chile (USD 800M loss to salmon aquaculture).

Highly potent fish-killers include the globally distributed, taxonomically unrelated dinoflagellate genera *Alexandrium, Karenia*, *Karlodinium* and *Margalefidinium,* raphidophytes *Chattonella* and *Heterosigma,* dictyochophytes *Pseudochattonella* and *Vicicitus,* and haptophytes *Chrysochromulina* and *Prymnesium.* All these species have in common their propensity to produce lytic compounds that irreparably damage the sensitive gill tissues of fish which ultimately die from suffocation. Except for recent advances with *Karlodinium* (karlotoxins), *Prymnesium* (prymnesins), and *Karenia brevisulcata* (brevisulcenals), the precise mechanisms of how such microalgae kill finfish remain poorly understood. Reactive Oxygen Species can be a co-factor in ichthyotoxicity, notably with raphidophytes such as *Chattonella*. While some species are always ichthyotoxic, others such as *Heterosigma, Pseudochattonella* and *Alexandrium catenella* kill fish only under certain conditions or life stages. Broad scale ecosystem impacts from fish killing algae are less common with raphidophytes and dictyochophytes that require intimate cellular contact for harmful effects, compared to *Karenia* and *Prymnesium* where intracellular or excreted toxins are responsible.

Critical hurdles that limit progress in our understanding of ichthyotoxins and their control and mitigation include: HABs at fish farms are not usually a research priority until a major bloom occurs; data sharing between industry and scientists is very limited; and there is a lack of standardized methods to detect ichthyotoxins in low concentrations dissolved in seawater. Currently, the RT fish-gill W1 (rainbow trout epithelial gill cell line) and *Chaetoceros* Quantum Yield bioassays are the most promising candidates for international standardization and intercalibration for some HABs.

The abundance of HABs that will adversely impact or kill fish is of considerable interest to fish farmers, open-water fishers, and natural resource management authorities. However, this varies with HAB strains and species, type and age of fish, but also local conditions of water temperature, salinity, turbulence and tidal flushing. Climate change also contributes to the unpredictability of fast fish killing blooms. Prevention, prediction and monitoring are no longer sufficient, but we actively need to pursue broad-scale tools to stop the blooms, for example by means of clay flocculation of algal biomass and/or targeted mopping up of ichthyotoxins.

We review existing knowledge and provide a roadmap for scientists, aquaculturists and insurance companies to improve management of fish-killing algal blooms that put pressure on seafood security for an ever-increasing human population.

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List of Acronyms

CGD	Complex Gill Disease
CORFO	Corporación de Fomento de la Producción de Chile
CREAN	Centro de Estudios de Algas Nocivas
FKA	Fish-Killing Algae, or Ichthyotoxic Algae
FKT	Fish- Killing Toxins, or Ichthyotoxins
GlobalHAB	Global Harmful Algal Blooms programme (IOC-UNESCO)
HAB	Harmful Algal Bloom
IOC	Intergovernmental Oceanographic Commission (IOC-UNESCO)
IFOP	Instituto de Fomento Pesquero
IPHAB	Intergovernmental Panel on Harmful Algal Blooms
UNESCO	United Nations Educational Scientific and Cultural Organization

Glossary

Allelochemicals. Bioactive compounds produced by a living organism that exert a detrimental physiological effect on individuals of another species when released into the environment. Some allelochemicals produced by HABs may also have ichthyotoxic potency (e.g. from *Alexandrium minutum*) but this does not apply to all ichthyotoxic HABs.

Chaetoceros Quantum Yield bioassay. Assessment of physiological stress of the easy to culture diatom strain *Chaetoceros muelleri* strain CCAP1010-3 when exposed to extracts, filtrate or lysate of potentially ichthyotoxic algae. This bioassay is based on the assumption that ichthyotoxins are the compounds measured. While this was confirmed for *Margalefidinium polykrikoides* and *Alexandrium minutum*, for most ichthyotoxic microalgal species this assumption still needs to be verified. Initially based on cell mortality and chlorophyll fluorescence assessment (Lelong et al. 2011), this bioassay was later refined by measuring maximum quantum yield (Fv/Fm) by pulse-amplitude-modulation (PAM) fluorometry (Long et al. 2018).

Cytotoxic. A broad term for any compound that is toxic to cells, including against cell lines such as mouse lymphoid P388 cells or neuroblastoma. Most cytotoxic compounds would also cause lysis of red blood cells (haemolytic) or damage fish gill membranes (ichthyotoxic).

Fish gill damage. The first point of attack by ichthyotoxic HABs are the fish gills, resulting in a generalized necrotizing degeneration of the epithelium of the secondary lamellae with associated sloughing. This is often accompanied by swelling and pyknosis of the primary lamellar epithelium and congestion of branchial vessels. Fish gills can only respond in a single way to ichthyotoxic challenge: different algal groups therefore produce comparable gill pathology, and this is similar across different fish species, such as rainbow trout, tuna, yellowtail fish, damselfish, sheepshead minnow etc. Once the fish gills are compromised, gill ventilation is impaired, and a loss of Bohr effect impacts on the blood haemoglobin's oxygen binding affinity. Furthermore, algal neurotoxins (if present) can penetrate the blood stream, and secondarily can cause fish behavioural changes.

Haemolytic. A broad term for any compound that causes lysis of red blood cells (erythrocytes), whether of mammalian (human, sheep, rabbit) or fish origin.

Ichthyotoxins, as used in the context of this document, are HAB-produced bioactive compounds that kill fish. The most common mechanism of killing fish is by damaging the sensitive gill membranes of fish (e.g. by karlotoxin, karmitoxin, prymnesins), but some compounds (e.g. brevetoxin, gymnodimine) are also thought to be absorbed via the gills and cause behavioural and cardiological problems for fish or even cause liver damage.

Marine mass mortality events, as used in the context of this document, are HAB-caused sudden deaths of thousands to millions of fish, that also often can impact a broad range of marine invertebrates. In some cases, non-toxic high biomass HABs can generate anoxic conditions and/or clog the gills of fish. The present report confines itself to ichthyotoxic HABs that damage fish gills either via direct cell contact (e.g. *Chattonella, Heterosigma*) or the production of ichthyotoxins (*Karenia mikimotoi, Prymnesium parvum*).

Neurotoxins are synthetic or naturally occurring substances that damage, destroy, or impair the functioning of the central and/or peripheral nervous system. HAB-produced neurotoxins include bre-

vetoxins, ciguatoxins, domoic acid and saxitoxins. Critically, many ichthyotoxins such as karlotoxins, karmitoxins and prymnesins are not neurotoxins and hence are not of human health concern.

Polyunsaturated fatty acids (PUFAs) are lipids in which the constituent hydrocarbon chain possesses two or more carbon-carbon double bonds. Free fatty acids (FFA) such as OPA (octadecapentaenoic acid, C18:5n3), EPA (eicosapentaenoic acid, C20:5n3), and DHA (docasahexaenoic acid, C22:6n3) have high ichthyotoxic potency (Okaichi 1983, Yasumoto et al. 1990). These compounds are prone to oxidative degradation, called lipid peroxidation, in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage.

Reactive Oxygen Species. Oxygen radicals are unstable molecules (half-life in the order of seconds) that readily react with other molecules to cause damage to DNA, RNA, proteins, and thereby cell death. Fish-killing raphidophytes, notably *Chattonella*, are potent producers of ROS. Ruptured algal cells consistently produce more ROS. However, superoxide and hydrogen peroxide on their own do not kill fish or cause fish gill damage, but ROS may serve as important co-factors for other ichthyotoxic compounds.

RT gill-W1 bioassay. RTgill-W1 is an epithelial cell line that was isolated from the gill of a normal rainbow trout *Oncorhynchus mykiss*. This cell line was deposited by NC Bols into the American Type Culture Collection (ATCC) and has been widely used to test organic chemicals with a wide range of physico-chemical properties for acute fish toxicity (e.g. OECD test guideline 249). It was adapted for HAB ichthyotoxicity assessment by Dorantes-Aranda et al. (2011), and been widely used since by laboratories in Australia, Canada, Chile, Denmark and France.

Sterolysins. A category of ichthyotoxins (e.g. karlotoxins, karmitoxin, amphidinols) that cause damage by binding to sterols such cholesterol in the lipid bilayer of gill membranes. This mechanism has not yet been demonstrated for prymnesins or brevetoxins.

Introduction

Gustaaf Hallegraeff

In the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae, about 41 species are responsible for algal blooms noxious to fish and other marine organisms, and others are producing toxic substances accumulated in shellfish, fish, and some other marine organisms. Discrimination between noxious bloom formers and toxin producers is difficult for some species, because some toxin producers may also kill fish, e.g. *Alexandrium* spp. Fish-killing harmful species include 22 dinoflagellates, 6 raphidophytes, 8 haptophytes, 3 dictyochophytes and 2 pelagophytes.

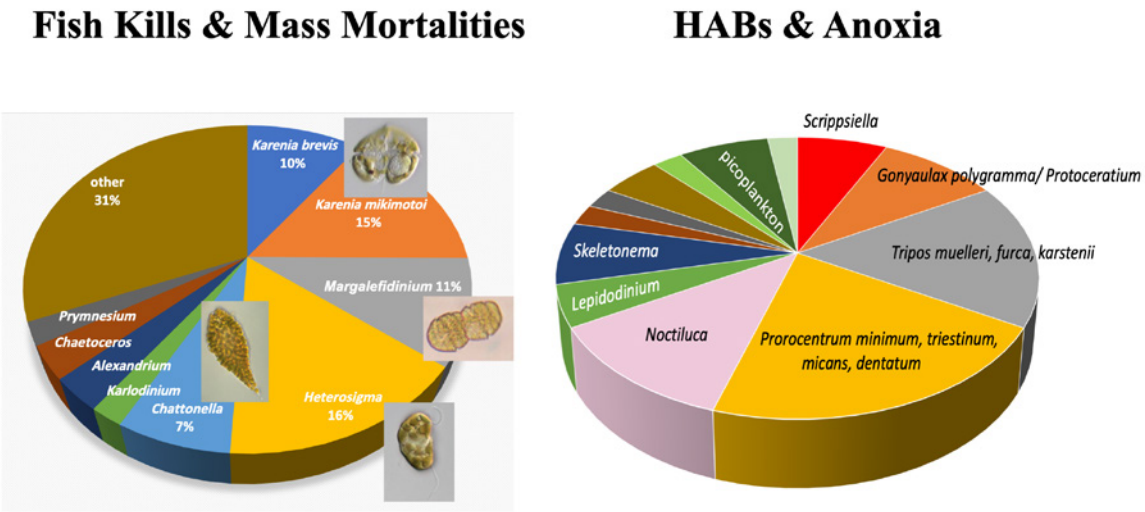

Figure 1.1. Overview of causative organisms of (left) fish kills and mass mortalities and (right) HABs and anoxia (from HAEDAT; as of December 2019).

A survey of 9,500 HAEDAT events (Figure 1.1) identified 7% of mass mortalities and fish kills, with the key causative organisms in order of decreasing importance being *Heterosigma* (16%), *Karenia mikimotoi* (15%), *Margalefidinium*/*Cochlodinium* (11%), *K. brevis* (10%), *Chattonella* (7%), and *Karlodinium, Alexandrium, Prymnesium, Chaetoceros* and others (including *Heterocapsa circularisquama*) contributing 31% of cases. Species responsible for anoxia (0.4% of total; Figure 1.1 right) included 70% dinoflagellates (*Gonyaulax polygramma, Protoceratium reticulatum, Prorocentrum micans, P. minimum, P. dentatum, P. triestinum, Noctiluca, Scrippsiella*), 17% diatoms and 13% others. Many more virulent fish-killers include the globally distributed, taxonomically unrelated dinoflagellate genera *Alexandrium, Karenia* and *Karlodinium, Margalefidinium*, raphidophytes *Chattonella* and *Heterosigma*, dictyochophytes *Pseudochattonella* and *Vicicitus*, and haptophytes *Chrysochromulina* and *Prymnesium*. These latter microalgal species and their impacts on finfish aquaculture, rather than shellfish or other biota, are the focus of this report.

From 8 to 11 October 2019 an Advanced International Colloquium and Technical Workshop was held in Puerto Varas, Chile, under the auspices of IOC-IPHAB and GlobalHAB, with the support of the government of Chile through CORFO and collaboration of CREAN-IFOP. Ten invited participants (D.M. Anderson, A.D. Cembella (working group chair, apologies), O. Espinosa, L. Guzman, G.M. Hallegraeff, P.J. Hansen (acting chair), H. Hégaret, T.O. Larsen, M. Iwataki, L. MacKenzie) reviewed state-of-knowledge and addressed gaps in knowledge to develop strategies for technological and scientific approaches

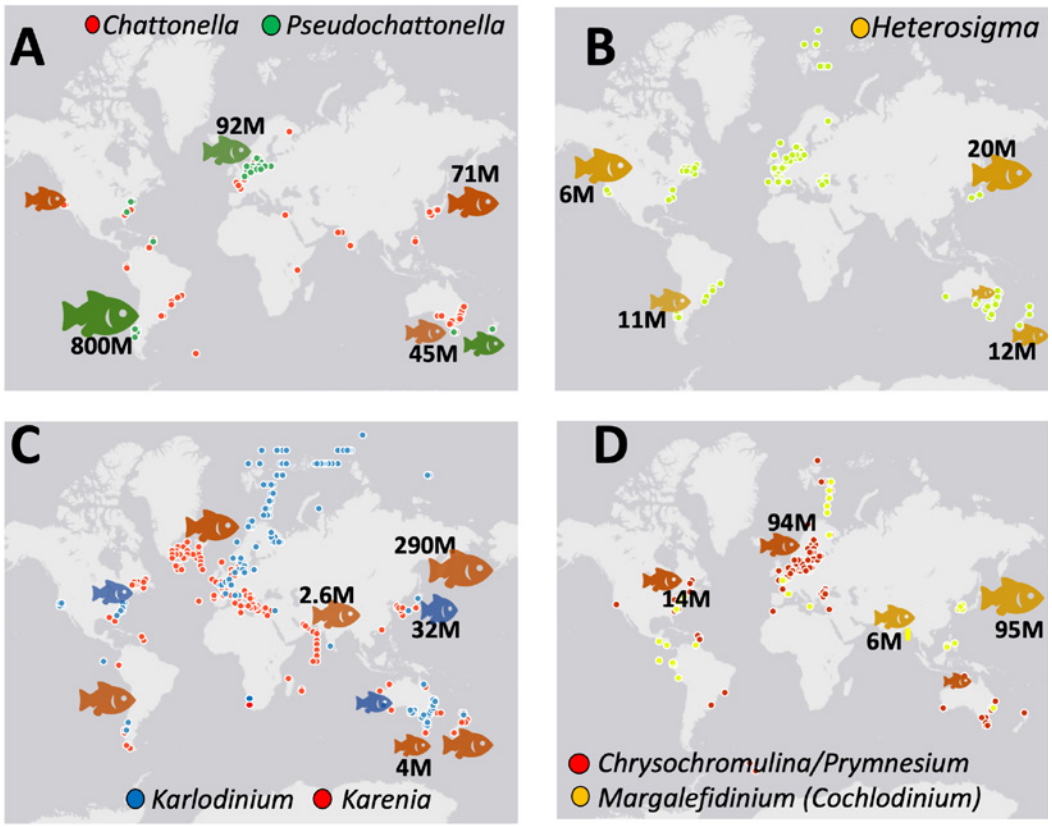

Figure 1.2. Known geographic distributions (from OBIS; as of December 2019) of key fish-killing HAB organisms (coloured dots): A. *Chattonella/ Pseudochattonella*, B. *Heterosigma*, C. *Karlodinium, Karenia* and D. *Prymnesium/ Chrysochromulina, Margalefidinium*. Fish icons represent exceptionally large economic impacts, indicated by the size of the fish and with economic impacts expressed as million USD damages. Large fish kill events require the incidence of the causative organisms, under favourable environmental conditions, but also the presence of vulnerable aquaculture operations.

to mitigate impacts. Additional contributors were solicited for their expertise following the workshop, including K. Davidson, F. Gianella and J.E. Rensel.

This report summarizes the issues addressed by the participants during the workshop structured according to six main themes: (1) Taxonomy; (2) Chemistry and analytical methods; (3) Modulation of ichthyotoxin production and release; 4) Mechanism of ichthyotoxin effects; (5) Which bioassays to select? (6) Ecology, physiology and role of climate change; (7) Ecosystem impacts; (8) Prevention, control and mitigation. The topic of (9) Socio-economics was added during the report writing stage.

In Chapter 2 on socio-economics the global scale of the ever growing finfish aquaculture industry is emphasized (90M t production, worth 138.5B per annum). Economic impacts from fish killing HABs are not confined to just loss of fish (up to USD 800M in Chile in 2016) but the further direct and indirect gross effect of blooms can be three times higher also driving changes in market price, consumer demand and employment in land-based service industries. HAB risks cannot yet be adequately quantified by insurance underwriters. This is where HAB research can help to better define probabilities of harm to allow for more confident insurance and hence better business planning. However, to achieve this we need more consistent monitoring approaches between different fish farm aquaculture companies, and sharing of data and potentially sensitive commercial information between industry, insurance companies and scientists to build trust and encourage collaboration.

Chapter 3 describes the taxonomic history, nomenclatural changes, and remaining questions on the identity of key species of fish-killing algae. Highly potent fish-killers include the globally distributed, taxonomically unrelated dinoflagellate genera *Alexandrium, Karenia*, *Karlodinium* and *Margalefidinium,* raphidophytes *Chattonella* and *Heterosigma,* dictyochophytes *Pseudochattonella* and *Vicicitus,* and haptophytes *Chrysochromulina* and *Prymnesium.* Most of them are fragile cells that call for better

preservation methods in routine monitoring programs. High precision molecular detection methods to distinguish for example between the different strains or chemotypes with differing ichthyotoxic potencies of *Prymnesium parvum, Heterosigma akashiwo* or *Pseudochattonella verruculosa* remain to be explored.

Chapter 4 summarises existing knowledge chemistry of microalgal toxins associated with mass fish killing events, which is limited because of the lack of lack of standardized methods to detect ichthyotoxins in low concentrations dissolved in seawater. Proven and chemically characterized ichthyotoxic compounds include karlo/karmitoxins, amphidinols and prymnesins which differ from other ladder-shaped toxins such as brevetoxins, brevisulcenals and gymnocins in possessing a lipophilic part of the molecule. To date, only karlotoxins have been confirmed to be present during fish kills in ecologically relevant concentrations, but this still need to be achieved for the other molecules, many of them (gymnocin, gymnodimine, brevisulcenals, brevetoxins) only kill fish at high concentrations. Co-factors such as reactive oxygen species (notably for *Chattonella*), free fatty acids and other lytic compounds (*Alexandrium*) are likely to be involved.

Chapter 5 focuses on the mechanism of fish kills. The mode of action of karlotoxins has been shown to be related to the disruption of the cell membrane by specific binding to cholesterol, thus creating pores in the membrane. Fish gill damage results in reduced respiratory and osmoregulatory capacity, hyperventilation and ultimately death from suffocation from internal oxygen deficiency. Ichthyotoxic algae are not always toxic to target cells and organisms. Below a certain threshold cell density, these algae have no effects. Furthermore, ichthyotoxins tend to disappear from the water quickly when the algae producing them are removed. Different target cells/organisms have different sensitivity to the ichthyotoxins.

Chapter 6 examines the wide range of bioassays that have been used to determine the potency of ichthyotoxic algal species, including whole organisms such as microalgae, *Artemia,* copepods, shellfish or fish (embryos, larvae or juveniles), such as rainbow trout, zebrafish or sheepshead minnow. Cell line

Figure 1.3. Participants at the Puerto Varas 2019 workshop, from left to right: O. Espinosa, L. MacKenzie, J. Mardones, M. Iwataki, T. Larsen, H. Hégaret, K. Yarimizu, L. Guzmán, P.J. Hansen, G. Hallegraeff, D. Anderson.

Table 1.1. Summary of iconic HAB impacts on finfish aquaculture in cases where economic impacts (in terms of fish lost) were estimated. * fish pond culture

HAB species	Country	Financial Losses/Year	Author
Chattonella marina/antiqua	Japan Japan South Australia Mexico	USD 71M, 1972 USD 53M, 2010 AUD 45M, 1996 80% of stock lost, 2016	Okaichi 1989 Sakamoto et al. 2021 Hallegraeff et al. 2021 Garcia-Mendoza et al. 2018
Pseudochattonella verruculosa	Chile Norway Denmark New Zealand	USD 800M, 2016 USD 1.4M, 1998 USD 1.4M, 2019 200 t (15% of stock), 2019	Mardones et al. 2021 Karlson et al. 2021 Karlson et al. 2021 MacKenzie et al. 2011
Heterosigma	Japan British Columbia New Zealand Chile Chile	USD 135M, 1980-90 USD 35M, 1980 NZD 12M, 1989 USD 11M, 1988 5000 t, 2021	Sakamoto et al. 2021 Rensel & Whyte 2014 Bousted et al. 1989 Mardones et al.2021 Mardones et al. 2023
Margalefidinium (Cochlodinium)	Korea Korea Korea Korea China Japan Canada	USD 69.5M, 1995 USD 19.5M, 2003 USD 10.5M, 2007 USD 22.5M, 2013 USD 2M, 1999 USD 40M, 2000 CAD 2M, 1999	Park et al. 2013 Park et al. 2013 Park et al. 2013 Park et al. 2013 Sakamoto et al. 2021 Sakamoto et al. 2021 Whyte et al. 2001
Karlodinium australe	Malaysia	USD 2.6M, 2014	Lim et al. 2014
Karlodinium (Karenia) digitatum	Hong Kong	USD 32M, 1998	Sakamoto et al. 2021
Karlodinium veneficum	US	38 t, 1996*	Deeds et al. 2002
Karenia mikimotoi	Norway Japan	USD 6M, 1988 USD 43M, 1984	Karlson et al. 2021 Sakamoto et al. 2021
Karenia longicanalis (=*K. umbella*)	Tasmania	AUD 4M, 2003	Hallegraeff et al. 2021
Chrysochromulina leadbeateri	Norway	USD 3.5M, 1991 USD 93.5M, 2019	Karlson et al. 2021 Karlson et al. 2021
Prymnesium polylepis	Norway	USD 9M, 1988	Karlson et al. 2021
Prymnesium parvum	Norway US	USD 9M, 1989 USD 18M, 2001*	Karlson et al. 2021 Roelke et al. 2011
Phaeocystis globosa	China	USD 26M, 1997-98	Sakamoto et al. 2021
Alexandrium catenella	Chile Chile Faroe Islands	USD 60M, 2002 USD 10M, 2009 27 t (77% of stock), 1984	Mardones et al. 2015 Mardones et al. 2015 Mortensen 1985

Table 1.2. Harmful phytoplankton species known or suspected of causing fish losses in aquaculture and recommended action levels. Updated after Rensel and Whyte (2003).

HAB species	Action level (cells/mL) to intensify management	References
Dinoflagellates		
Alexandrium catenella	250-300; 300-500; 397-975	Mardones et al. 2015; Haig 2016, Montes et al. 2018
Margalefidinium polykrikoides	300 to 1,000; 3300	Kim 1998; Yuki and Yoshimatsu 1989; Tang and Gobler 2009
Karenia brevis	5-10; >10-25; 2500	Steidinger et al. 1998
Karenia mikimotoi	500 to 2,000; 1,000 to 3,000	Kim 1998; Tangen 1977; Rensel and Whyte 2003
Karlodinium australe	2340	Lim et al. 2014
Karlodinium veneficum	60,000 (striped bass); 115,000,000 (juvenile cod)	Nielsen 1993; Place et al. 2012
Haptophytes		
Chrysochromulina leadbeateri	1000-2000	John et al. 2022
Prymnesium polylepis	1000; 4000-8000	Rensel and Whyte 2003; Haigh 2016
Prymnesium parvum	10,000-20,000 (striped bass, palmetto bas, Tilapia); 500,000	Shilo 1982, Roelke et al. 2011
Raphidophytes		
Chattonella antiqua *C. marina*	50 -66 (tuna); 500 (yellowtail, seabream); up to 500,000; dependent on fish species, size, HAB strain	Okaichi 1989; Hallegraeff et al. 1998; Haigh 2016; Garcia-Mendoza et al. 2018
Heterosigma akashiwo	>50, less if exceedingly calm and warm weather; highly variable from non-toxic to >750-1,000	Rensel and Whyte 2003; Rensel 2007; Rensel et al. 2010
Dictyochophytes		
Pseudochattonella verruculosa	>1 -50 (salmon Chile); 10 (salmon New Zealand); 90 (Europe)	Montes et al. 2018; MacKenzie et al. 2011

bioassays, such as mammalian or fish cell lines or microalgal cultures offer important experimental advantages of faster results, simplicity, cost-effectiveness and specificity, and their use has increased, especially in western countries to comply with increasing ethical regulations on animal experimentation. The RT gill-W1 (rainbow trout epithelial gill cell line) assay has proven to be the most promising standardized method, but it must be kept in mind that the response of this cell line may not necessarily represent the complex response of the whole fish. The fish-gill bioassay is complicated to implement in the field or on a boat, but has been successfully applied to water samples transported within 12-24 h to a shore-based lab. An alternative could be the diatom *Chaetoceros muelleri* QY (quantum yield) bioassay), since the PAM fluorometer can be carried in the field and requires low amounts of material.

Chapter 7 looks at broader ecosystem effects which are common for fish-killing blooms where intracellular or excreted toxins are primarily responsible (e.g. *Karenia* spp. and *Prymnesium/ Chrysochromulina* spp. blooms). However, ecological effects appear less common with blooms of raphidophytes (e.g. *Heterosigma)* and dictyochophytes (e.g. *Pseudochattonella*) that require intimate cellular contact for harmful effects. On the one hand, HABs can cause significant negative environmental effects and the destruction of valuable fisheries resources. On the other hand, they may be analogous to wild fires, periodically thinning out populations, maintaining species diversity and enhancing resilience.

Chapter 8 summarises existing knowledge on the ecophysiology of key fish-killing HAB taxa. Some species respond to nutrients as the key driver (*Karlodinium, Prymnesium, Chattonella*), most HABs show higher growth rates at higher temperatures (with exception of the cold-water *Alexandrium catenella* and *Karenia selliformis*). Being flagellates, nearly all are favoured by increased water column stratification. *Heterosigma* and possibly *Pseudochattonella* also respond to salinity stratification. High biomass does not necessarily mean high ichthyotoxicity, however; e.g. phosphorus deficiency appears to drive toxicity of *K. brevis*, *K. veneficum* and *P. parvum*. A major gap in our knowledge is how lytic compounds will respond to changes in temperature, pCO_2 and nutrient (N, P, Fe) availability.

The final **Chapter 9** discusses options for prevention, control and mitigation, which ideally all require prior knowledge on the HAB species involved. For example, aeration sometimes can lyse fragile algal cells, perimeter skirts only help against surface slicks, and deep upwelling calls for knowledge of depth distribution of HABs. While clay flocculation initially focused on removing algal cell biomass, targeted applications of using modified clay for ichthyotoxin adsorption can be achieved at clay loadings, significantly lower than those considered to be harmful to benthic marine invertebrates (roughly equivalent to two packets of sugar spread over one square meter). HABs are considered disasters, and in most countries, the public, politicians and aquaculture industry expect some level of HAB control to mitigate their impacts.

The questions raised here constitute a "roadmap" for the next 10 years for scientists, stakeholders and policy makers to improve management of fish killing algal blooms that put pressure on seafood security for an ever-increasing human population.

Socio-economics of finfish aquaculture

Keith Davidson and Fatima Gianella

Global world aquaculture fish production is now effectively equal to that of wild capture fisheries at 90M tonnes per annum (FAO 2022a, b) and predicted to continue increasing. Brown et al. (2020) estimate that finfish aquaculture contributes 54M tonnes of this production with a value of USD 138.5B. For some countries, aquaculture has become a central pillar to their economy. For example, the value of the industry in Norway, the largest global producer of farmed Atlantic salmon, was estimated in 2018 to be USD 8.3B (OECD 2021). In Scotland, the 3rd largest global salmon producer, the industry had a turnover of USD 1.87B in 2018. This is approximately 1% of the country's gross domestic product, with production and the subsequent supply chain generating an economic gross value added of USD 1.11B (Biggar Economics 2020). In Scotland, the industry supports 12,000 jobs that are of particular importance to the remote rural locations in which it is primarily situated. Given its significant economic scale sustaining and expanding farmed fish, this production is critical to both local and national economic strategy in many of the major salmon-producing countries.

A number of studies have attempted to evaluate the economic impact of HABs. Examples include Hoagland et al. (2002), who analysed several other marine sectors, with subsequent research addressing commercial fisheries (Hoagland and Scatasta 2006; Jin and Hoagland 2008; Jin et al. 2008), recreational fisheries (Hoagland and Scatasta 2006; Dyson and Huppert 2010), tourism (Hoagland and Scatasta 2006; Taylor and Longo 2010; Morgan et al. 2011), health (Hoagland et al. 2014, Diaz et al. 2019), seafood markets (Wessels et al. 1995) and shellfish aquaculture (Rodriguez et al. 2011, Martino et al. 2020). Brown et al. (2020) estimated the economic impacts of HABs on aquaculture amount to approximately USD 8B/yr globally. But in addition to mass mortalities of finfish, this value includes harvesting bans on the sale of shellfish that have accumulated unsafe levels of HAB phycotoxins and costs related to human health, with no compartmentalisation of losses between different categories.

The paucity of HAB economic studies specifically related to finfish aquaculture is caused by the lack of available data. Economic impacts are determined in a counterfactual analysis, i.e. comparing with the outcomes should the HAB event not have happened (Jin et al.2020). Such analysis requires economic data on factors such as production and mitigation costs, which are often confidential in an aquaculture setting. Moreover, there is often also a lack of available phytoplankton and environmental data. In contrast to shellfish farming, which in many countries is highly regulated with published monitoring data related to HABs and their biotoxins, as well as information on harvesting closures and production yields, there is little comparable information for finfish aquaculture. Many fish farms conduct their own HAB monitoring, but these data are rarely publicly available. Furthermore, data related to fish health are considered sensitive for aquaculture businesses and not often disclosed. This lack of available data means that independent studies on the HAB-fish farm economic link in the peer reviewed academic literature or elsewhere are lacking (Martino et al. 2020).

Analysis of economic impacts from HABs requires information on the timing, duration, and geographic scope of a HAB event, as well as time-series measures of economic activities in each affected region and sector (e.g., value of fish mortalities; Jin et al. 2020). More sophisticated analyses require additional information. In the context of fish cage aquaculture, these include spatial or company specific

data on HAB monitoring and management practices, and more detailed data on economic activities, e.g., costs of mitigation measures or "clean up" costs, and ideally, data on the impact of the HAB events on the supply chain.

As with all farming, maintaining healthy stock is critical to the sustainability of a business. It is clearly in the interests of aquaculture producers to minimise losses from both an animal health and economic point of view. Data from Salmon Scotland indicate that about 1.3% of farmed salmon die each month, giving an annual survival rate of 85.5%. While 15% mortality per year may be lower than the mortality rate that wild salmon experience at sea (Chaput 2012), mortalities can generate a significant cost and negative public perception of industry. Health standards are ensured through regulation with fish health inspectors visiting farms on a regular basis to ensure best practices are undertaken. However, neither inspectors nor farmers can prevent naturally occurring HAB events in the same way as, for example, good husbandry practices can limit bacterial, fungal, or viral fish diseases.

While ichthyotoxicity from phytoplankton taxa, such a raphidophytes, is perhaps the most well-known cause of farmed fish mortality (Peperzak 2002), the physical impact of other taxa, for example spiney diatoms (Bruno et al. 1989) on the gill health of farmed fish is increasingly of concern for the fish farming industry. The problem is further complicated by multiple health challenges experienced by farmed fish. These include HABs but also viral and fungal pathogens that all contribute to complex gill disease (CGD; Herrero et al. 2018). This combination of factors impacting fish health and survival makes it difficult to quantify their individual economic impact.

The economic impacts of HABs can be chronic as well as acute, with the former scenario being related to promotion of low or medium level CGD based fish health issues. While largely unquantified, the cost of HABs and/or their contribution to CGD will include the costs of any mitigation measures such as aeration, oxygenation, increased monitoring of HABs, and, where regulations allow, moving fish to waters with reduced HAB abundances. Other costs include those associated with removal and disposal of dead fish, and in the event that ongoing HAB issues leads to large changes in salmon aquaculture production, the consumer price is potentially affected (Adams et al. 2018). In extreme cases, CGD/HABs may also lead to job losses or temporary closures on the land-based side of the aquaculture sector, which can then impact sales and jobs in related industries.

Acute, mass mortality events are more easily characterized. As an example, the 2019 bloom of *Chrysochromulina leadbeateri* in northern Norway that was estimated to have killed 8 million salmon, total tonnage 14,000, had a direct economic cost of over USD 93.5M. This was compounded by a future

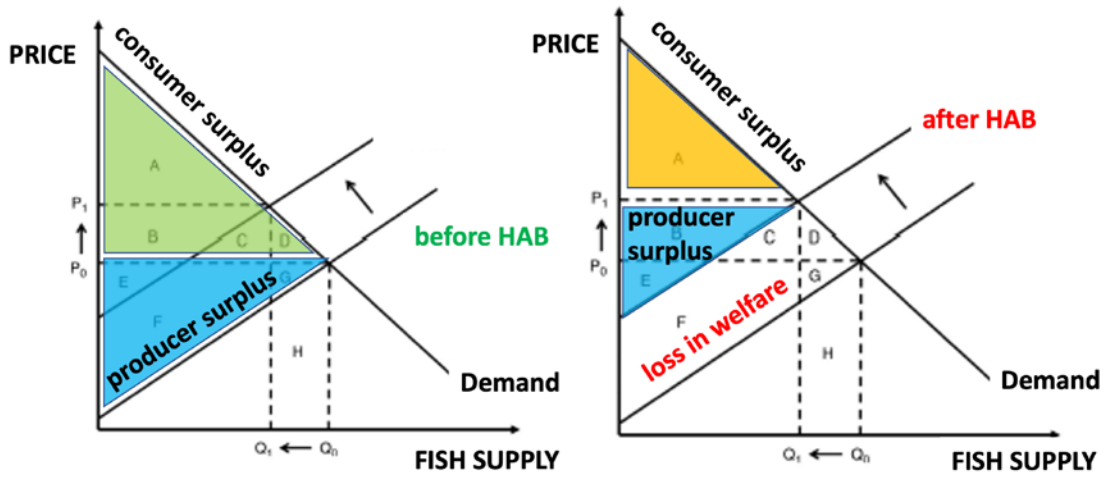

Figure 2.1. Economic welfare impacts (changes in fish price) between (left) a non-HAB and (right) a HAB event causing mortality in fish farms. The fish supply curve declines from Q_0 to Q_1 while prices increase from P_0 to P_1. True economic losses comprise reductions in both consumer and producer surpluses. For the initial market equilibrium (left), areas A+B+C+D (green triangle) represent the benefit for the consumers,

sales loss of approximately the same amount, as the fish were not fully grown out for sale. Furthermore, clean-up cost and extra mitigation cost added up to a further USD 33M, with additional loss of tax income and requirements to fund unemployment/social benefits. The direct and indirect gross effects of the bloom have therefore been estimated to be between USD 253M and 308M, which is three times the economic loss from the killed fish alone (Kontali 2020). Figure 2.1 illustrates how a HAB event can trigger economic changes via a shift in the supply of the industry (reduction in fish production) and a change in price (increase) even if consumer demand is not affected by the HAB. It shows how direct impacts (measured as lost revenue) are different from measures of consumer and producer surplus. Benefits for consumers are measured under the demand and above the market price curves, while measures of surplus for the producers are the area above the supply and below the market price curves. In case of large HAB impacts that introduce volatility in supply, consumer price is also affected as well as employment in land-based service industries.

Economic techniques now exist to better evaluate the impact of HABs for smaller fish kills and sub-lethal events (Adams et al. 2018, Davidson et al. 2020). These methods also serve to provide guidance on the application of mitigation actions, providing some confidence on the net benefits of such strategies can be guaranteed. However, the various HAB/economics studies conducted to date are not easily comparable, because they are based on different metrics (Davidson et al. 2014). Hence, should further studies of the economic impacts of HABs on finfish aquaculture be developed, it will be important to include both the direct economic losses from mortalities and sub-lethal effects, as well as indirect economic impacts of mass mortalities of farmed fish for society.

Even if data relating to HABs and fish health are available, evaluating the "costs" to finfish aquaculture businesses of a HAB is more challenging than for other forms of aquaculture as it is difficult to stipulate threshold concentrations above which a particular HAB taxon may be of concern. This "threshold" approach is successfully applied to the management of many shellfisheries, with harvesting halted or toxin testing increased if a harmful species exceeds a particular abundance. However, no consensus of thresholds above which an ichthyotoxic species would be considered harmful exist. This is partly because of the lack of historical data on HAB abundances at fish farms preventing any statistical analysis on their relationship with health. However, gill health in farmed fish can be impacted by a range of challenges and hence the fish health consequences of particular abundances of a HAB event may vary depending on the age of the fish and whether experiencing, or has recently experienced, any other health challenges (for example sea lice infestation, amoebic gill disease, viral infection).

The lack of standardised monitoring practices for HAB taxa between finfish aquaculture practitioners is a serious limitation to understanding and mitigating HAB issues, and hence in addressing their economic impact. Monitoring of HAB taxa at fish farm sites is not universal, but when undertaken it usually occurs daily and is based on "live" cell counts using a conventional light microscope. Cells may be collected by net haul or bottle, but with no universally accepted protocols. The lack of cell fixation and cell settling, that is commonly used in HAB monitoring at shellfish farms, reflects the requirement for rapid operational decision making and that some important fish killing taxa (such as raphidophytes) do no survive the fixation process. However, this approach makes cell identification and quantification difficult, particularly for fish farm staff that are rarely highly trained taxonomists and who have a range of other husbandry tasks to undertake on a daily basis. Cross comparison of data from different companies, or even farms in the same company, is also problematic as target species and taxonomic nomenclature is often based on "in house" practices.

In an effort to address the inconsistencies in HAB monitoring in the farmed fish sector, a standardised phytoplankton monitoring framework has recently been developed for Scottish waters (Weeks et al. 2022). This responds to a need identified by the Scottish Government led Farmed Fish Health Framework (FFHF) for coherence in collection, identification and reporting of potentially harmful marine phytoplankton at finfish aquaculture sites. The monitoring framework provides guidelines for aqua-

culture companies on standardised sampling protocols, species lists and data formats for reporting that are intended to provide consistency across the Scottish finfish sector. Taxonomic training is also being developed to upskill farm staff and promote best practice and consistency of approach. If widely adopted, this standardisation of practice will enable enhanced understanding of phytoplankton risks and challenges, including better understanding of the impact of HAB events on gill and overall fish health. It will also enhance the potential for centralised reporting and the opportunity for aquaculture practitioners to exchange standardised information that provides early warning of developing events. If operated collectively, for example through an interactive web-based portal (such as the *www.HABreports.org* system for Scottish waters), this approach has the potential to minimise the economic consequences of HABs in a particular region, particularly if combined with predictive mathematical modelling over timescales of days and weeks (Davidson et al. 2021).

Daily monitoring of HAB taxa at farm sites is relatively expensive in staff time. This cost to a company of an enhancement in monitoring must therefore be balanced against the relative lack of mitigation measures available should a HAB event be identified. The lack of clear strategies to deal with HABs even if monitoring or modelling provides early warning of their appearance has therefore led some companies to simply build HAB losses into their financial models. Improved methods of management and mitigation are therefore critical for the sector to better deal with the HAB challenge (Figure 2.2).

Insurance offers aquaculture businesses the potential to mitigate the financial impacts of HABs. Jin et al. (2020) cites Lim and Kim (2020) as a case study of the Korean aquaculture disaster insurance system that was instigated in 2008 to compensate for damages to the aquaculture sector caused by natural disasters including HABs. By end of 2018, 4,250 (44.3%) of South Korean aquaculture farms were insured, with 60% of their premium being subsidized by government (Lim and Kim 2020). Elsewhere, including the finfish aquaculture sector of major producing countries, insurance costs are typically borne solely by the producer. Moreover, not all marine insurers offer suitable cover and others may set large premiums, as the HAB risk cannot be appropriately quantified by underwriters and hence there is limited competition in the market.

Gianella (2023) compiled available data related to the economic compensation from major HAB mediated farmed fish-based insurance claims in eight countries (UK, Norway, Ireland, Iceland, Faroe Islands, Chile, Canada and Australia) over the period 2010-2021 (Figure 2.3A). The annual number of

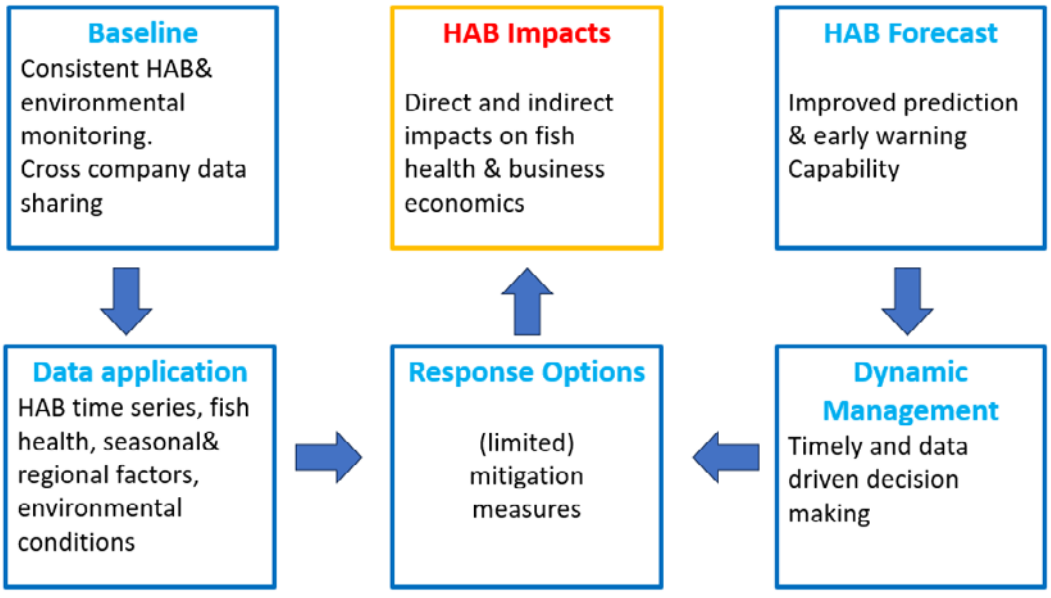

Figure 2.2. Key components of HAB socioeconomic analysis and their relationships for fish farming, adapted from Jin et al. (2020).

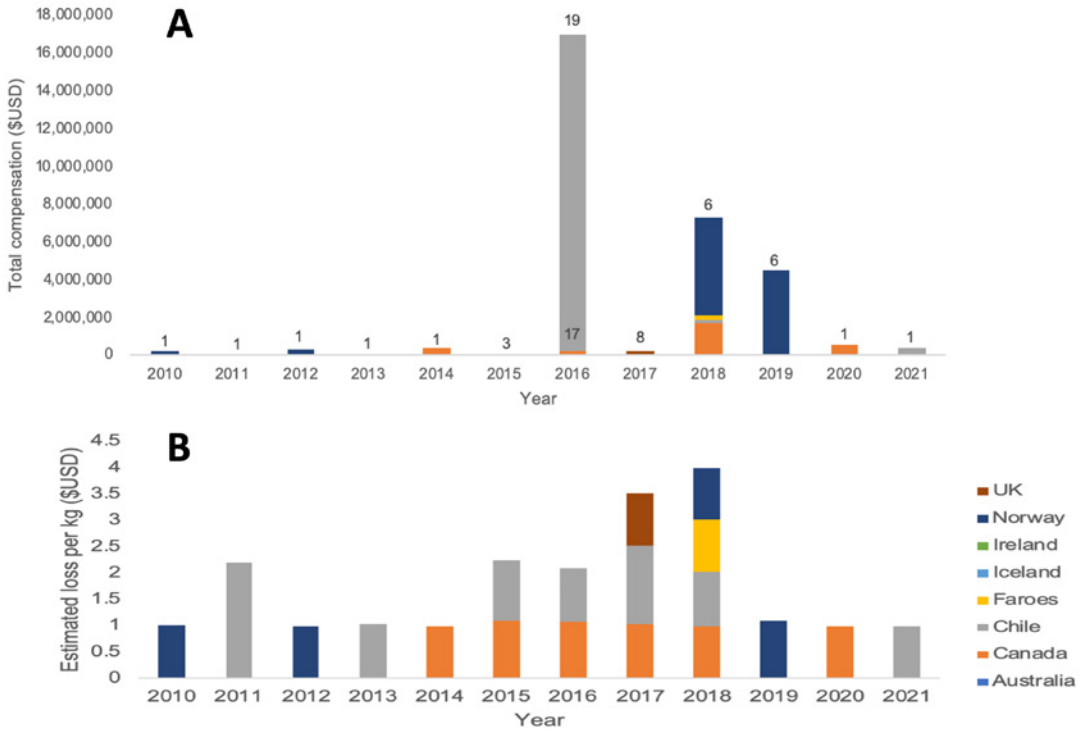

Figure 2.3. Insurance metrics from a single insurance company covering fish farming in eight global countries in the period 2010 - 2021; A. Total economic compensation (USD) and total number of claims (numbers above bars); B. Economic loss estimation (USD) per kg of salmon. From Gianella (2023).

claims and economic compensation were variable throughout the time series and within countries. Losses were few in the first five years of the data series, only related to Norwegian (2010, 2012) and Canadian (2014) fish farms. The highest economic loss occurred in 2016, related to the extraordinary *Pseudochattonella* event in Chile (Mardones et al. 2021). This generated an insurance loss of USD 17M from the 17 claims in Chile in that single year. The absence of claims in the first period of the time series and increasing number of claims and total compensation in the last five years supports the industries' perception of an intensification of HABs. However, this should be carefully assessed, as whether these data depict a change in HAB frequency or a change in take up of insurance is unclear. Moreover, the data series is short and contradicts worldwide decadal data that claim a relationship between intensification of monitoring efforts and more frequent bloom reports (Hallegraeff et al. 2021; Karlson et al. 2021).

Diverse insurance approaches may be applied depending on the natural hazard and sector covered. These are classified as probabilistic (catastrophe insurance), parametric (environmental variable based) and deterministic (bespoke for specific sites) approaches. Fish farms are predominantly insured using a deterministic approach, assessed according to the specific site, technology used and worker experience. The catastrophe modelling approach focuses on estimating the potential losses following a natural disaster, and hence has potential for application to major HAB events. However, to set a premium an underwriter requires historical data and scientific understanding to inform models that estimate the annual probability of occurrence of the hazardous event. It is here that HAB research can help to better define probabilities of harm to allow for more confident insurance and hence better business planning.

Gianella (2023) also estimated economic loss per kilogram of salmon (USD/kg) as a combination of total of compensation payments, clean-up cost, number of fish lost, and average weight of fish. Average weight of mature salmon was assumed constant at 2.5 kg, during their first year at sea after the freshwater culturing stage (Munro 2021; insurance company pers. comm.). Compensation was estimated at USD 3.00 per lost fish over 2.5 kg. Clean-up costs were assumed to be equally divided

between insured and non-insured costs (that were therefore met by the farm). The estimation also incorporated a deductible proportion (20% of fish mortality), that reflects typical mortality in the total production cycle (Kenyon and Davies 2018). Total economic loss per kg over the 2010-2021 time series for the eight countries covered was calculated using the following equation.

$$\text{Estimated economic loss per kg} = \frac{\text{Total fish mortality}}{\frac{\text{(Total payment + clean up cost)}}{\text{Average weight fish (2.5 kg)}}}$$

Economic loss per kilogram of fish was variable across the time series but relatively similar in different countries (Figure 2.3B). The lowest accumulated losses are typically related to years with lower numbers of claims (2010, 2012, 2013, 2014). However, in 2011 and 2015, years of low economic compensation and a total of only one and three claims respectively led to USD 2.2 annual loss per kilo each year. The year 2018 displayed the highest accumulated economic loss per kilogram of farmed salmon (~USD 4/kg) with HAB events in multiple countries. The similar estimated economic loss could be related to the use of the deterministic insurance approach and mitigation measures taken by the insurance market, for instance, implementing a cap on claims in Chile after the 2016 incident events hence limiting insurance company exposure to HAB losses. Information and collaboration with the fish farms is needed to better clarify the total economic losses due to HAB occurrence.

Priority topics for research and implementation

- Better understand the interaction between HABs and other health challenges to fish.
- More consistent monitoring approaches between different fish farm aquaculture companies to ensure data quality and transferability.
- Better sharing of monitoring data and potentially sensitive commercial information between industry, insurance companies and scientists to build trust and encourage collaboration.

Taxonomy and identification of fish-killing harmful algae

Mitsunori Iwataki

Unambiguous identification of harmful microalgal species is essential for understanding their distribution, ecophysiological traits, production of bioactive compounds and bloom formation. Taxonomic knowledge also is critical for effective HAB species monitoring to mitigate fisheries damages by fish-killing harmful algae. Diagnostic traits for microalgal taxonomy are group specific, e.g. armored dinoflagellates have been described mainly based on the number and arrangement of thecal plates covering the cell, and unarmored dinoflagellates have been described based on characters of the cingulum and apical groove. Morphology assessed by light microscopy has traditionally been used for description of microalgae, and ultrastructure assessed by electron microscopy has provided further characters for taxonomy, such as organic body scales in haptophytes (e.g. *Chrysochromulina* and *Prymnesium*) and some dinoflagellates (e.g. *Heterocapsa*), and apical groove of unarmored dinoflagellates (e.g. *Gymnodinium, Karenia* and *Karlodinium*). In the last two decades, molecular-based phylogeny has been increasingly applied to their classification. This has led to several new genera being proposed for fish-killing microalgae, e.g. dinoflagellates *Karenia* and *Karlodinium* (Daugbjerg et al. 2000), *Takayama* (de Salas et al. 2003), *Margalefidinium* (Gómez et al. 2017), and dictyochophytes *Pseudochattonella* (Hosoi-Tanabe et al. 2007) and *Vicicitus* (Chang et al. 2012). These morphological and molecular data provide better solutions for microalgal taxonomy and identification. In this section, taxonomic history and status of major fish-killing microalgae are summarized for a better understanding of the taxonomic changes and remaining questions to be addressed.

3.1. Raphidophyte *Chattonella*

The marine raphidophyte *Chattonella* Biecheler has caused fish mass mortalities in temperate to tropical regions (Hallegraeff et al. 2021; Sakamoto et al. 2021; Yñiguez et al. 2021). *Chattonella* is a naked flagellate with two (anterior and posterior) flagella and many brownish chloroplasts peripherally located (Hallegraeff and Hara 2004), and the cell is relatively larger than other marine raphidophytes such as *Heterosigma* and *Fibrocapsa*, although species identification is difficult. The genus *Chattonella* was originally described from the Mediterranean with the type species *C. subsalsa* in Biecheler (1936). The second species *Hornellia marina* Subrahmanyan was reported from the southern coast of India (Subrahmanyan 1954), and later transferred to *Chattonella* as *C. marina* (Subrahmanyan) Hara et Chihara (Hara and Chihara 1982). The large species *Hemieutreptia antiqua* Hada was described from the Seto Inland Sea, Japan, and later recognized as a species of the genus, as *C. antiqua* (Hada) Ono (Hada 1974; Ono and Takano 1980). Subsequently, four other *Chattonella* species, *C. globosa* Hara et Chihara, *C. minima* Hara et Chihara, *C. ovata* Hara et Chihara, and *C. verruculosa* Hara et Chihara were described (Hara et al. 1994). Of these, *C. globosa* and *C. verruculosa* have since been transferred to the Dictyochophyceae inferred from rDNA sequences, as *Vicicitus globosus* (Hara et Chihara) Chang and *Pseudochattonella verruculosa* (Hara et Chihara) Tanabe-Hosoi, Honda, Fukaya, Inagaki et Sako, respectively (Hosoi-Tanabe et al. 2007; Chang et al. 2012). These molecular studies based on LSU rDNA and ITS regions showed monophyly of the genus *Chattonella* in the Raphidophyceae, and that the *Chattonella* clade separated into at least two subclades representing *C. subsalsa* and *C. marina* (Lum et al. 2021). The morphospecies *C. antiqua* and *C. ovata* are included in the *C. marina* clade, and

Demura et al. (2009) recognized these two species as varieties within *C. marina*. According to Hara and Chihara (1982), *C. marina* resembles *C. subsalsa* but differs in the absence of oboe-shaped ejectile mucocysts and presence of thylakoid penetration into the pyrenoid matrix. However, mucocysts were observed also from specimens in the *C. marina* clade (Branco et al. 2019), and thylakoid penetration into the pyrenoid matrix was found also in *C. subsalsa* (Klöpper et al. 2013). *Chattonella marina* and *C. subsalsa* currently cannot be distinguished based on these characters, and therefore other morphological characters separating these species are needed. Two new species *C. tenuiplastida* (slim chloroplasts) and *C. malayana* (curving bean-shaped chloroplasts) were recently described based on chloroplast fine structure and molecular sequences (Lum et al. 2022). Another problem with current *Chattonella* phylogeny is the lack of DNA sequences for *C. marina* from the location of original description from south India.

3.2. Dictyochophyte *Pseudochattonella*

The dictyochophyte *Pseudochattonella* is the causative species of the world's largest farmed fish-killing algal bloom in Chile, 2016 (Mardones et al. 2021). Two *Pseudochattonella* species have been described and both are recognized as fish-killing microalgae, *P. verruculosa* and *P. farcimen* (Eikrem, Edvardsen et Throndsen) Eikrem. The former was originally described as *Chattonella verruculosa* from Japan and later transferred to the genus *Pseudochattonella*, and the latter was described as *Verrucophora farcimen* Eikrem, Edvardsen et Throndsen from Norway and later combined into the earlier established genus *Pseudochattonella* (Hara et al. 1994; Edvardsen et al. 2007; Hosoi-Tanabe et al. 2007; Eikrem et al. 2009). Cells of *Pseudochattonella* resemble *Chattonella*, being ellipsoid with a slightly tapering posterior end and two flagella inserted at the anterior but have numerous mucocysts protruding on the cell surface. Discrimination of the two *Pseudochattonella* species is difficult due to their morphological variability. Cell lengths are reported as 5-50 µm for *P. farcimen* and 12-45 µm for *P. verruculosa* (Hara et al. 1994; Edvardsen et al. 2007). According to Edvardsen et al. (2007), *P. farcimen* forms long cells which was not reported from *P. verruculosa*, and grows in 5-10 °C and does not tolerate >15 °C, whereas *P. verruculosa* shows maximal growth at 17 °C (Yamaguchi et al. 1997; Skjelbred and Naustvoll 2006).

3.3. Unarmored dinoflagellates *Karenia, Karlodinium* and *Takayama*

Species of the unarmored dinoflagellate genera *Karenia, Karlodinium* and *Takayama* have caused fish-killing blooms worldwide (Anderson et al. 2021; Hallegraeff et al. 2021; Sakamoto et al. 2021; Sunesen et al. 2021; Yñiguez et al. 2021). Fish-kills by *Karenia brevis* (Davis) Hansen et Moestrup, *K. mikimotoi* (Miyake et Kominami ex Oda) Hansen et Moestrup, and *Karlodinium veneficum* (Ballantine) Larsen have long been reported, and noxious blooms of other species such as *K. longicanalis* Yang, Hodgkiss et Hansen, *K. selliformis* Haywood, Steidinger et MacKenzie, *Karlodinium digitatum* (Yang, Takayama, Matsuoka et Hodgkiss) Gu, Chan et Lu, *Takayama acrotrocha* (Larsen) de Salas, Bolch et Hallegraeff have been responsible for fisheries damage for the last two decades. Other kareniacean species are probably ichthyotoxic when they form blooms, e.g., a bloom of *Karlodinium australe* de Salas, Bolch et Hallegraeff was reported with fish-kills in Johor Strait in Malaysia, 2014 (Lim et al. 2014). Unarmored dinoflagellates have traditionally been classified based on the position and distortion of the cingulum, according to Kofoid and Swezy (1921), and species described before the establishments of kareniacean genera belonged to *Gymnodinium* or *Gyrodinium*. Daugbjerg et al. (2000) demonstrated the phylogenetic separation and morphological characters of *Karenia* and *Karlodinium* species, distinct from *Gymnodinium* and *Gyrodinium*, and proposed two new genera. Subsequently, *Takayama* was established for species having a sigmoid apical groove (de Salas et al. 2003). Since the species in *Karenia, Karlodinium* and *Takayama* have characters distinct from *Gymnodinium* and *Gyrodinium* sensu stricto, such as the chloroplasts containing fucoxanthin and the straight or sigmoid apical

groove, molecular phylogeny has helped to construct their natural classification. New species of these genera have been increasingly described with molecular-based phylogenetic information (e.g. *Karlodinium decipiens* de Salas). As for the kareniacean dinoflagellates described without molecular data, the molecular phylogenetic positions were determined for some species (e.g., *Karenia longicanalis*), but were not established for others (e.g., *Takayama pulchella* (Larsen) de Salas, Bolch et Hallegraeff).

In the genus *Karenia*, two noxious bloom species, *K. brevis* and *K. mikimotoi*, were originally described as *Gymnodinium breve* Davis and *G. mikimotoi* Miyake et Kominami ex Oda, respectively (Oda 1935; Davis 1948). For this genus, *K. brevis*, *K. brevisulcata* (Chang) Hansen et Moestrup, and *K. mikimotoi* were transferred by Daugbjerg et al. (2000) and several species later were described with molecular data, e.g., *K. umbella* de Salas, Bolch et Hallegraeff (de Salas et al. 2004). Wang et al. (2018) examined DNA sequences of freeze-dried bloom specimens from Hong Kong in 1998, including *K. digitata* and *K. longicanalis*, and the results showed that the former was positioned in *Karlodinium* and combined as *Karlodinium digitatum* (Yang, Takayama, Matsuoka et Hodgkiss) Gu, Chan et Lu. The latter was closely related to *K. umbella* de Salas, Bolch et Hallegraeff, and therefore *K. umbella* is now recognized as a junior synonym of *K. longicanalis*. Moreover, recent molecular studies showed the affinities of *Brachidinium capitatum* Taylor and *Asterodinium gracile* Sournia to *Karenia* (Henrichs et al. 2011; Benico et al. 2019). Since these genera have been described earlier than *Karenia,* that is *Brachidinium* by Taylor (1963) and *Asterodinium* by Sournia (1972), *Brachidinium* has priority. Conservation of the genus *Karenia* against *Brachidinium* and *Asterodinium* is required to prevent further name changes of this important harmful algal taxon and any unwanted confusion.

Karlodinium is small kareniacean dinoflagellate with straight apical groove, and is distinguished from *Karenia* based on the presence of the ventral pore and plug-like structure underlying the amphiesma (Daugbjerg et al. 2000). *Karlodinium micrum* (Leadbeater et Dodge) Larsen is the type species but is currently recognized as *Kl. veneficum*. Bergholtz et al. (2005) re-defined *Karlodinium* by the chloroplasts containing internal lenticular pyrenoids and the presence of a ventral pore. Several small *Karlodinium* species such as *Kl. ballantinum* de Salas, *Kl. gentienii* Nézan, Chomérat et Siano, *Kl. zhouanum* Luo et Gu were described based on their separation of phylogenetic position from *Kl. veneficum*; they closely resemble each other and are only discernable with help of molecular sequences (Benico et al. 2020). *Takayama* species are characterized by their sigmoid apical groove, which differs from the straight apical groove of *Karenia* and *Karlodinium*. This genus was established with the type species *Takayama tasmanica* de Salas, Bolch et Hallegraeff and *T. helix* de Salas, Bolch, Botes et Hallegraeff, with three previously described species *T. acrotrocha* (Larsen) de Salas, Bolch et Hallegraeff, *T. cladochroma* (Larsen) de Salas, Bolch et Hallegraeff and *T. pulchella* based on their sigmoid apical groove (de Salas et al. 2003). *Takayama* species have formed fish-killing blooms but unambiguous identification is difficult due to size and morphological variations. Two distinct clades have so far been recognized in the genus, one includes *T. tasmanica* and the other include species tentatively classified as *T. acrotrocha* (possibly synonymous with *T. xiamenensis*; Lu *et al.* 2022). For reliable classification of the genus, size and morphological variation in each *Takayama* clade should be examined. Furthermore, molecular data of previously described species, *T. acrotrocha, T. cladochroma* and *T. pulchella*, should be obtained from the type localities for unambiguous identification.

3.4. Unarmored dinoflagellates *Margalefidinium* (*Cochlodinium polykrikoides* and *C. fulvescens*)

Fish-killing blooms of two unarmored dinoflagellates of *Margalefidinium, M. polykrikoides* (Margalef) Gómez, Richlen et Anderson and *M. fulvescens* (Iwataki, Kawami et Matsuoka) Gómez, Richlen et Anderson, have been reported from North and Central America, East Asia, and Middle East (Anderson et al. 2021; Sakamoto et al. 2021; Sunesen et al. 2021). These two species were originally described in the genus *Cochlodinium*, as *C. polykrikoides* Margalef from Puerto Rico and *C. fulvescens* Iwataki,

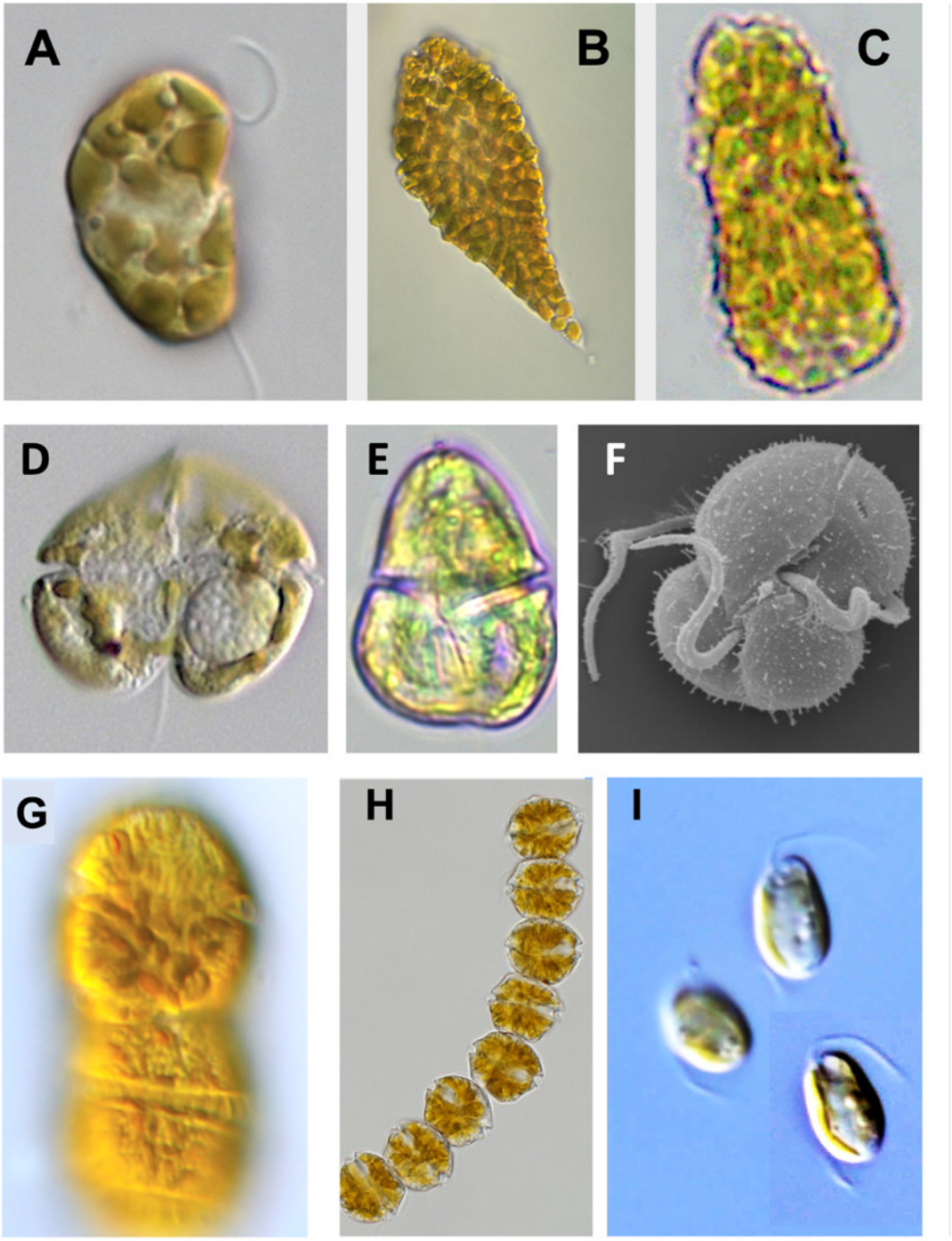

Figure 3.1. Fish-killing algae a. *Heterosigma akashiwo*, 15 µm long; B. *Chattonella marina*, 60 µm long; C. *Pseudochattonella verruculosa*, diameter 20 µm; D. *Karenia papilionacea*, diameter 20 µm; E. *Karenia selliformis*, diameter 30 µm; F. *Karlodinium veneficum*, diameter 15µm; G. *Margalefidinium polykrikoides*, 35 µm long; H. *Alexandrium catenella*, diameter 35 µm; I. *Prymnesium parvum*, 10 µm long. Photo credits A, D, F: M.de Salas; B, E, I: G.Hallegraeff; C, H: J. Mardones; G: M. Iwataki.

Kawami et Matsuoka from Japan (Margalef, 1961; Iwataki et al. 2007). New combinations in the newly established genus *Margalefidinium* were made due to their phylogenetic positions distant from the type species *Cochlodinium strangulatum* (Schütt) Schütt (Gómez et al. 2017). Like other unarmored dinoflagellates, *Cochlodinium* had been distinguished from other genera based on the character of the cingulum, encircling the cell >1.5 times. These two species form cell chains, and the cells share morphological characters such as the cingulum surrounding the cell >1.5 times of the cell, shallow sulcus on the cell surface, and an eyespot at the dorsal side. Moestrup (2020) pointed out that the conservation of the name *Cochlodinium polykrikoides* would have prevented confusion and name changes.

3.5. Morpho-molecular characterization of fish-killing microalgae

For the past 20 years, rDNA-based molecular phylogeny has been increasingly used for a better understanding of natural classification of fish-killing microalgae, and several new genera, such as *Karenia, Karlodinium, Takayama, Margalefidinium, Pseudochattonella* and *Vicicitus*, have been established for the species with phylogenetic positions incongruent with former morphotaxonomic classification. DNA sequences have been determined also for species originally described without molecular data, and some species were transferred to other genera in consideration of their phylogenetic positions (e.g. *Karlodinium digitatum*). Since the microalgal species are described based on morphological characters, both morphological and molecular characterization are important.

Priority topics for research and implementation

- Obtain molecular data for type specimens of fish-killing HAB species, and if not available, specimens from the type locality should be considered. This includes efforts to obtain material from the type locality of *Chattonella marina* from India and *Takayama pulchella* from Australia for further investigation.
- Explore toxin composition or other substances as informative tools in chemotaxonomy for the purpose of delineating HAB species. For instance, should different types of prymnesins produced by *P. parvum* be considered in species delineation?
- Explore novel preservation methods and high-precision molecular detection methods for fish-killing harmful algae.

Chemistry and analytic methods - how to deal with the many congeners?

Thomas Larsen

4.1. Fish-killing toxins

It is striking how little is still known about the chemistry of microalgal toxins associated with the massive fish killing events that have occurred through the past decades. This is in sharp contrast to microalgal toxins that have been demonstrated to be toxic to humans, most of which accumulate in shellfish (Figure 4.1). From a chemical ecological point of view, one could speculate that fish-killing toxins have not evolved to kill fish, since microalgae live in fierce competition with other prokaryote and eukaryote microorganisms at the microscale in marine environments. It is more plausible that

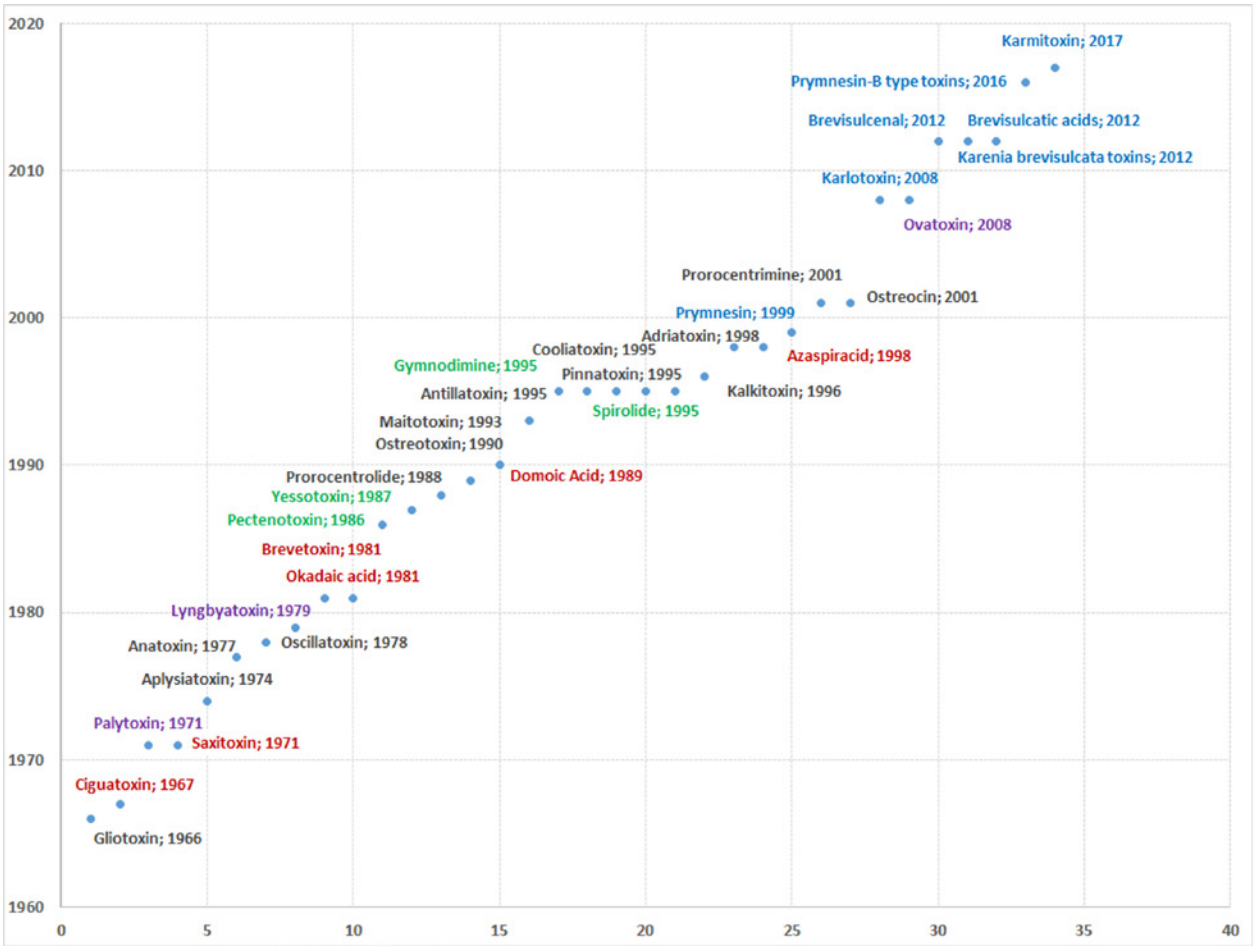

Figure 4.1. Historic overview of discovery and description of the structure of the first analogues of 34 microalgal toxin groups (1966-2017). Color code: blue, toxins involved in fish kills; red, toxins involved in human poisoning; violet, toxins causing skin irritation or respiratory problems (brevetoxins involved in fish kills, human poisoning and skin irritation or respiratory problems); green, toxins known for 20-30 years and not proven to have negative effects on humans or aquatic organisms; black, compounds yet to be related to effects in humans or aquatic biota (adapted from Hess 2018).

mixotrophic microalgae such as Karlodinium produce lytic compounds used for lysis of cell membranes of the microorganisms that they feed on (Place et al. 2012).

A common feature of many of fish-killing microalgal species seems to be their capability to produce polyketide derived lipophilic lytic compounds that binds into the lipid bilayer of the membranes of sensitive fish gill tissues. It has been proposed that this mechanism of action for karlotoxins includes important binding interactions with cholesterol; accordingly, these compounds are sometimes referred to as sterolysins (Waters et al. 2015; Figure 5.1). The unique 4α-methyl sterol profiles by *Karlodinium* provide a protective mechanism against self-intoxication, in contrast to their known prey species that possess des-methyl sterols (Adolf et al. 2006). This may also be the case for prymnesins.

Other mechanisms, such as Reactive Oxygen Species (ROS) have been speculated to be a co-factor in ichthyotoxicity, notably likely for raphidophytes such as *Chattonella* and *Heterosigma,* but also for dinoflagellates such as *Margalefidinium* and *Karenia* (Table 4.1). While species in some genera are always ichthyotoxic, others such as *Pseudochattonella, Heterosigma* and *Alexandrium* (e.g. *A. catenella)* cause problems only under special conditions.

In many cases, it has not been clearly documented which toxins are causing a given massive fish killing algal bloom, even when there is no doubt about the identity of the bloom-forming organism. One example is the coupling of prymnesin 1 & 2 originally described by Igarashi et al. (1996) from *Prymnesium parvum*. Despite the full structural elucidation of prymnesin-1 and 2, they were not detected by other researchers during numerous HAB events for almost two decades. It was only with the structural characterization of a slightly shorter B-type of prymnesins, and as a yet unknown C-type of prymnesin (Rasmussen et al. 2016b), including the fact that many congeners of all three types exist, that the complexity of these toxins became evident (Binzer et al. 2019). In other cases, reporting of specific toxin production in relation with a HAB event has been disputed. This includes production of brevetoxins by raphidophytes, claimed by Onoue et al. (1990) but disputed by McNabb et al. (2006). Brevetoxins have so far have only been unambiguously detected in Florida strains of *Karenia brevis*, but their quantitative role in causing fish kills remains to be demonstrated since purified PbTx2,3 exhibited limited ichthyotoxicity against RTgill cells (Dorantes-Aranda et al. 2015) and only in high concentrations. This also applies to brevisulcenals from *K. brevisulcata*, gymnocin from *K. mikimotoi* and gymnodimine from *K. selliformis,* because no suitable analytical methods are available to monitor and assess their concentrations during fish kills in nature. With *K. selliformis*, some non-gymnodimine producing strains also cause ichthyotoxicity (Mardones et al. 2021), emphasizing our lack of knowledge regarding chemistry of FKTs.

4.2. Chemistry, biosynthesis and structural diversity of fish killing toxins

Most known FKTs belong to the group of polyketides, an extremely diverse group of compounds produced by a wide range of organisms, ranging from prokaryotes, unicellular algae to higher eukaryotes (Anestis et al. 2021). In contrast to eukaryotic microorganisms such as filamentous fungi (Wang et al. 2022) and bacteria, much fewer details are currently known about the underlying mechanisms for polyketide derived biosynthesis of FKTs. However, recent studies have demonstrated that unique modular type I polyketide synthases (PKSs) are involved in the biosynthesis of polyketide derived polyethers in both dinoflagellate and haptophyte species such as *Gambierdiscus polynesiensis* (Kohli et al. 2017) and *Prymnesium parvum* (Anestis et al. 2021), respectively. Due to their structural differences polyether FKTs have been categorized into different subclasses such as 1) linear superchain polyethers (karlotoxins, amphidinols, karmitoxins); 2) ladder-frame polyethers (brevetoxins, ciguatoxins, yessotoxins, gymnocins), and 3) supersized ladder-like polyethers (prymnesins, brevisulcenals, maitotoxins) (Rasmussen et al. 2016a).

Intriguingly, species in genera that are not closely related may still produce structurally highly similar ichthyotoxins, clearly indicating an evolutionary relationship between such species. This is in particular exemplified by the karlo-/karmi-toxins produced by species in the dinoflagellate genus *Karlodinium*, which resemble the amphidinols and related compounds produced by *Amphidinium carterae* (Rasmussen et al. 2016a). On the other hand, it is also evident that different species within a genus do not produce the same congeners of a given toxin type. This is exemplified by species in genus *Karlodinium*, where *K. veneficum* produces karlotoxins, whereas *K. armiger* so far has been the only species described to produce a linear superchain polyether containing an amino group (Mooney et al. 2009; Place et al. 2012; Rasmussen et al. 2016a; Binzer et al. 2019).

Minor structural differences have even been observed among strains within an apparently well-defined microalgal species (Rasmussen et al. 2016b). More specifically, three different types of prymnesins (type A, B and C-prymnesins) are produced separately by three clearly distinct clades within *Prymnesium parvum* (Binzer et al. 2019). Accordingly, a given *P. parvum* strain only produces one of these three types of prymnesins, however often including numerous congeners of the given prymnesin type. Moreover, the toxin profiles seem to be conserved within a certain strain irrespective of culture conditions (Anestis et al. 2021; Medic et al. 2022). It has been speculated that evolution of similar types of genes encoding modular type 1 PKS's in *P. parvum* have evolved by gene duplications into A-, B- and C-type prymnesins (Binzer et al. 2019). In this way, an original C-type prymnesin with an 83-carbon long backbone might have evolved into the longer B-type (85 carbon atoms), by addition of just one extra acetate extender unit to the initial growing polyketide chain, followed by further gene duplications, leading to A-type prymnesins with the currently longest backbone of 91 carbon atoms. This hypothesis is in agreement with the phylogenetic results obtained from ITS and rDNA sequencing of a total of 26 strains indicating that strains producing the C-type prymnesins represent the ancestral type of *P. parvum* (Binzer et al. 2019). It is tempting to speculate

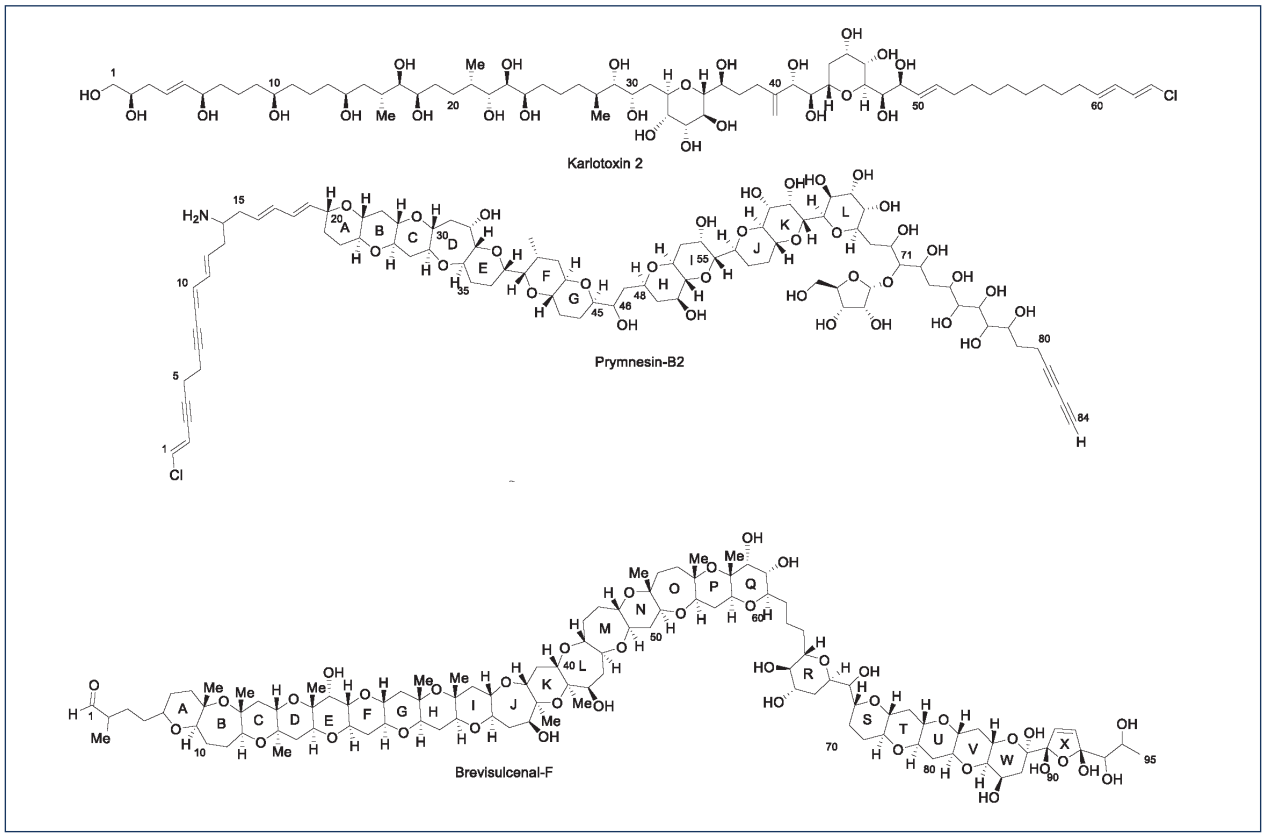

Figure 4.2. Chemical structures of selected microalgal fish killing toxins (Rasmussen et al. 2016a).

that the A-, B- and C-type prymnesin-producing strains indeed represent three different, but closely related species evolved from the same ancestor.

4.3. Analytical methods and challenges

The past decade has revealed that the major known types of FKT's are often produced as a mixture of numerous structurally diverse, but on the other hand, very similar compounds with small variances in e.g. chlorination, saturation or attached sugar moieties. A prominent example of this are the prymnesins where so far 51 congeners have been reported on top of the three that have been fully structurally characterized (Binzer et al. 2019). It is even more challenging to purify standards and to develop methods/tools to quantify the concentrations of these compounds for early warning systems. Other notable examples for which numerous congeners have been described recently are karlotoxins/amphidinols/karmitoxin and brevisulcenals (Rasmussen et al. 2016a).

In the absence of pure standards for all congeners of a given class of FKTs, LC-MS/MS based methods have been developed for sensitive detection of compounds such as brevisulcenals (Harwood et al. 2014), karlotoxins (Bachvaroff et al. 2008; Krock et al. 2017) and amphidinols (Wellkamp et al. 2020). Validated sample preparation is the important fundament for all quantification methods, including recovery studies of ideally each variant of a given type of compounds to be analyzed for. In the case of brevisulcenal (KBT) and brevisulcatic acid (BSX) toxins this includes solid phase extraction (SPE), which was demonstrated to concentrate these toxins from algal extracts and seawater, importantly including acidification of cultures/seawater allowing stronger wash conditions to be applied eliminating unwanted matrix components (Harwood et al. 2014). Based on the availability of standards of four KBTs and four BSXs the authors were able to develop a quantitative triple quadrupole-based LC-MS/MS method for their quantification. By the use of relative response factors, the method furthermore included semi-quantitative analysis of further four BSX toxins for which no standards were available. The methods limit of quantification was determined to be 2-5 ng mL^{-1}, depending on the given toxin to be detected. Krock et al. (2017) used a similar LC-MS/MS based approach for detection and quantification of four known karlotoxins and one amphidinol, for which they had standards, using selected reaction monitoring (SRM). This approach is based on measurement of transition of the $[M+Na]^+$ adduct generated by electrospray ionization to the most abundant fragment, which in this case was quite distinct. The limit of detection for the specific karlotoxin KmTx-2 was 2.5 ng on-column in their case corresponding to 0.1 pg $cell^{-1}$ on a cellular basis with 10^6 cells extracted (Krock et al. 2017). The authors discuss that the fact that one million cells are needed to reach the LOD of their method, argues against the possibility of detecting karlotoxins in field samples with low or background concentrations of *Karlodinium* cells. In many ways this illustrates the challenges that we are facing with detection of FKTs in natural samples. Krock and other co-workers have also recently developed at similar LC-MS/MS based methods for quantification of known as well as detection of several new variants of amphidinols (Wellkamp et al. 2020).

A different analytical strategy has been used for indirect quantification of the supersized ladder-frame polyether prymnesins based on the fact that all three types contain a primary amine at C-14 of the lipophilic side chain (Figure 4.2). Accordingly, the method is based on derivatization of this amine group with a fluorescence tag and using two other primary amine containing compounds (fumonisin B_1 and B_2) as a proxy for the quantification (AccQ-Flour reagent). Since other compounds with primary amine groups will also be derivatized and detected, enrichment of extracts and fractions should be analyzed by liquid chromatography coupled to mass spectrometry in parallel in order to confirm the presence of prymnesins in the original sample (Svenssen et al. 2019).

Table 4.1. Summary of fish killing algae, their proposed ichthyotoxic mechanisms, impact on fish and other marine biota and references to laboratory trials on ichthyotoxins.

HAB species	Proposed Ichthyotoxic Mechanism	Fish affected
Alexandrium catenella	High molecular weight >5kDa lytic compound (Ma et al. 2011); synergism between ROS and DHA (Mardones et al. 2015).	Salmon.
Chattonella marina/antiqua	FFA in synergism with ROS; intimate contact of algae with fish gills required.	Yellowtail, seabream, bluefin tuna.
Heterosigma	ROS; brevetoxin-like compounds?; intimate contact of algae with fish gills required; more toxic when cells are intact.	Coho salmon, chinook salmon, Atlantic salmon.
Karlodinium armiger	Karmitoxin	No data.
Karlodinium veneficum	Karlotoxin and analogues.	Striped bass. Larger fish more susceptible.
Karenia brevis	Brevetoxins. Also thought to be absorbed across the gill membranes.	Atlantic tarpon, mullet, snapper, perch, grouper, kingfish, sharks, rays, eels
Karenia brevisulcata	Brevisulcenals, brevisulcatic acids.	Eels, flounder, yellow-eyed mullet, barracouta, mackerel, leather jacket, stargazers, kahawai.
Karenia mikimotoi	FFA; gymnocin A, B; gymnodimine; limited role for ROS (strain specific); intimate contact of algae with fish gills required.	Pacific/Atlantic/chum salmon, turbot, sea trout, rainbow trout, blennies, gobies, conger eel, butterfish, flat fish, cod.
Karenia selliformis	Gymnodimine?	Salmon
Margalefidinium polykrikoides	ROS, DHA, ODA.	Snapper Lutjanus guttatus, sheepshead minnow, red seabream, yellowtail, flounder.
Prymnesium parvum	Prymnesins 1&2 (Igarashi et al. 1996; Rasmussen et al. 2016a).	Striped bass, palmetto bas, Tilapia, salmon, barramundi, carp, eels.
Pseudochattonella verruculosa	Lytic compounds are released?	Salmon, sea trout, Atlantic menhaden. Mainly affecting larger fish.

Other biota impacted	Controlled laboratory studies
No	Surf smelt *Hypomesus japonicus* exposed to filtered dinoflagellate culture media died within 3-5 h (Ogata and Kodama 1986).
No	High ROS producer (Oda et al. 1997), but ROS on its own does not kill fish. FFA such as OPA and EPA killed damselfish at 3ppm, but ichthyotoxicity increased 15-fold in synergy with ROS (Marshall et al. 2003).
No	Ichthyotoxic to juvenile rainbow trout, but significantly reduced when superoxide dismutase and/ are catalase were added to mop up ROS (Yang et al. 1995).
Not available.	LC50 of 0.14 µg/ml of karmitoxin against RTGill cells, but sheepshead minnow larvae were 3x less sensitive (Binzer et al. 2020).
Mussels.	Juvenile cod *Gadus morhua* exposed to 1.15x108 cells/ml died in 2 days (Nielsen 1993). LD50 of 0.5 µg/ml for karlotoxins against larval sheepshead minnow and zebrafish (Deeds et al. 2006; Mooney et al. 2010) compares with concentrations during natural fish kills.
Oysters, crabs, birds, manatees, bottle-nose dolphins.	Exposure of mullet *Mugil cephalus* to intact cells @ 4,000 cells/mL caused no fish death, but exposure to lysed cells (68 ng PbTx-3/ml) killed fish within 10min (Naar et al., 2009. Algal cultures @ 180 cells/mL killed juvenile salmon with a LT50 of 0.75h (Shi et al., 2012). Purified PbTx-2 and PbTx-3 showed ichthyotoxic activity against RT gill cells only at high concentrations (LC50 of 22.1 vs 35.2 µg/mL) (Dorantes-Aranda et al. 2015).
Sea slug, starfish, sea urchin, abalone, seaweed	BSX and KBT are cytotoxic against mouse leukemia P388 cells (Satake et al. 2018, 2021). Algal cultures @ 10,000 cells/mL killed juvenile salmon with LT50 of 0.48h (Shi et al. 2012). Salmon more sensitive than snapper.
Abalone, squid, brittle stars, lugworms, pearl oyster, cockles, mussels, scallops, whelks, worms, sea urchins, sea cucumber, lobster.	Gymnocin A, B is cytotoxic against mouse lymphoid P388 cells, but 250x less potent against freshwater fish Tanichthys than brevetoxin (reviewed by Li et al. 2019). Gymnodimine is ichthyotoxic against Tanichthys at 0.1ppm (Seki et al. 1995).
Sea urchins, octopus, whelks, chitons, bi-valves, clams.	Non-gymnodimine producing strains can still be ichthyotoxic to RTgill-W1 cells. Gymnodimine showed ichthyotoxicity against *Tanichthys albonubes* at 0.1ppm (Seki et al. 1995). Causes gill and liver damage in medaka fish (Liu et al. 2023)
Oyster larvae, hard clam, coral reefs.	Lipid peroxidation in gill tissues of flat fish in proportion to cell density (Kim et al., 1999; Dorantes-Aranda et al., 2009). Peroxidase and catalase reduced ichthyotoxicity (Tang and Gobler 2009).
Tadpoles, molluscs.	Prymnesins killed *Tanichthys albonubes* fish at 3nm (Rasmussen et al. 2016b).
No	40-45% loss in viability of RTgill-W1 and CHSE-214 cell lines @ 100 cells/mL (Mardones et al., 2019; Sandoval-Sanheza et al. 2022). Inability to reproduce high ichthyotoxicity observed in nature (Andersen et al. 2015, Skjelbred et al. 2011).

Due to the above-mentioned challenges with detection and quantification of FKTs, alternative methods, such as immunoassays have emerged. These assays represent lower cost methods for rapid screening of routine samples due to their high sensitivity and lack of need for advanced instrumentation and highly educated personnel (Samdal et al. 2019). Using this approach, a practical ELISA-based method has been developed for detection of azaspiracids with a limit of quantification of 37 µg/kg for AZA-1 in shellfish. Importantly, this ELISA-based technique displayed broad cross-reactivity towards both azaspiracid reference materials as well as their precursors. The current Norwegian headed research project ToxANoWa is aiming at development of antibodies towards prymnesins.

4.4. Reactive Oxygen Species

The role of Reactive Oxygen Species (ROS) in ichthyotoxicity has long been suggested from whole fish experiments with *Heterosigma* and *Margalefidinium*, respectively, where application of ROS mopping enzymes such as catalase and peroxidase significantly improved fish survival (Yang et al. 1995, Tang and Gobler 2000). Oxygen radicals are unstable molecules (half-life in the order of seconds) that readily react with other molecules to cause damage to DNA, RNA, proteins, and thereby cell death. Fish-killing raphidophytes, notably *Chattonella*, are potent producers of ROS (Oda et al. 1997). Ruptured algal cells consistently produced more ROS (Marshall et al. 2005). Several other fish-killing algae, such as *Karenia* and *Alexandrium catenella,* are also strong producers, but for the weak ROS producers of *Heterosigma, Karlodinium* and *Prymnesium,* ROS on its own cannot be invoked. Using a xanthine-xanthine oxidase chemical reaction to generate superoxide at concentrations equivalent to fish-killing *Chattonella*, Marshall et al. (2003) demonstrated that superoxide on its own does not kill fish. Similarly, Twiner and Trick (2000) also showed that hydrogen peroxide produced by *Heterosigma* did not explain ichthyotoxicity. ROS also exhibited negligible impact (<14% decrease in viability) in the RT fish gill assay. This points to the fact that ROS can be a co-factor in combination with other bioactive compounds such as polyunsaturated fatty acids (PUFA) but which on their own cannot explain ichthyotoxicity (Marshall et al. 2013, Kim et al. 1999).

Okaichi (1983) investigating *C. marina/antiqua* blooms killing yellowtail in the Seto Inland Sea focused on free fatty acids damaging fish gills. This was pursued by Arzul et al. (1995) trying to understand fish-killing *Karenia mikimotoi* blooms. The latter workers identified free fatty acids such as OPA (octadecapentaenoic acid) and EPA (eicosapentaenoic acid) as having the highest ichthyotoxic potency. Mardones et al. (2016) also confirmed the ichthyotoxicity by DHA (docasahexaenoic acid) from *Alexandrium catenella*. In whole fish experiments both Sola et al. (1999) and Marshall et al. (2003) demonstrated that exposure to OPA and EPA, respectively, did cause fish gill damage at concentrations in seawater of approximately 3 ppm. This is short of what dense algal blooms would generate (1.5- 2 ppm). Pursuing the role of ichthyotoxicity by EPA, Marshall et al. (2003) demonstrated that when damselfish were challenged with EPA in the presence of ROS, this increased the potency of EPA by up to 15-fold. Similarly, DHA in synergism with ROS became 9 times more ichthyotoxic (Mardones et al. 2015). Synergisms between OPA and ROS, or OTA and ROS were less pronounced (Mooney et al. 2011).

A role for saxitoxin and analogues has often been implied in fish kill events by *Alexandrium catenella*. Natural fish kill events however consistently revealed very low STX accumulation in fish gills, muscle, brain and inner organs (<4 µg STX eq./100g wet wt), but at the same time noticeable oedema, hyperplasia and necrosis of secondary gill lamellae. Mardones et al. (2015) found no evidence for a role of STX in fish gill damage, but which instead could be explained by the production of unknown lytic compounds or synergism between DHA and ROS (Long et al. 2021).

In conclusion, we need better monitoring tools for detection and quantification of not only the FKTs for which their structures have been fully elucidated, but also their many uncharacterized congeners. Apart from dedicated analytical methods based on HPLC coupled to mass spectrometry, this should

also include development of new antibody-based methods with broad specificity towards individual classes of toxins, such as has recently been demonstrated for the shellfish associated azaspiracids (Samdal et al. 2019).

Priority topics for research and implementation

- Bioassay-guided identification of the unknown toxins from major fish killing species (e.g. *Pseudochattonella* and *Chrysochromulina*), for which we currently have no knowledge about their chemical nature.
- Characterize the structures of new FKTs by modern NMR techniques, as well as their physical chemical properties (e.g. stickiness), setting the scene for development of new analytical tools targeting both single toxins, and classes of toxins found as numerous congeners.
- Produce reference standards of major toxins for community sharing.
- Develop new antibody-based methods with broad specificity towards individual classes of toxins.
- Implement the use of metabolomics, looking at toxin profiles to study the broader distribution of key toxin classes among both closely and distantly related species in important microalgal taxa and genera.
- Implement open access sharing of chemical data of the known toxins, such as MS/MS spectra in Global Natural Product Social (GNPS) Networking repositories.
- Increase our knowledge about bioaccumulation and toxicity of FKT's for humans.

Mechanisms of effect and modulation of ichthyotoxin production and release

Per Juel Hansen

Ichthyotoxins, like prymnesins, karlotoxins, karmitoxins and amphidinols affect cell membranes leading to cell lysis (Rasmussen et al. 2016b; Svenssen et al. 2019; Binzer et al. 2019). Similar lytic effects have been observed with other not yet chemically characterized compounds from a number of other fish killing algae belonging to dinoflagellates, haptophytes, raphidophytes and dictyochophytes.

The first sign of the fish being affected by ichthyotoxins is that they produce mucus. Later, the fish will snap for air at the water-air interface. At some point, the fish will lose buoyancy and perform erratic bursts of swimming. The latter is usually a point of no return (Svendsen et al. 2017). Fish will die fast (minutes to hours) when exposed high cell densities of an ichthyotoxic alga (Yariv et al. 1961; Svendsen et al. 2017; Bergsson et al. 2019). At lower algal cell densities the fish reacts by producing

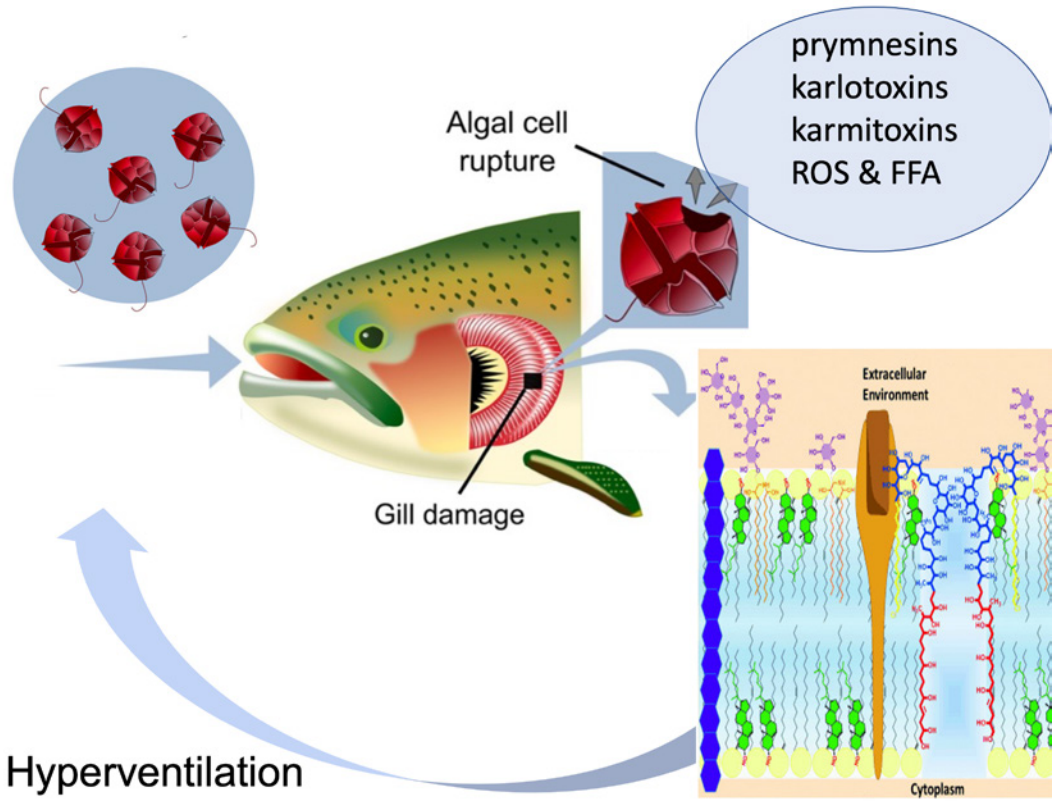

Figure 5.1. Schematic diagram summarising possible mechanisms of fish mortality when exposed to ichthyotoxic HABs. Microalgal cells are brought into contact with the sensitive fish gill lamellae through ventilation. Cell contents released from HABs include known ichthyotoxic compounds such as prymnesins and karlotoxins but also reactive oxygen species and free fatty acids. One mechanism of action of gill damage is based on studies of karlotoxin that cause pore formation in sterol containing cell membranes (Waters et al. 2015). Fish gill damage results in reduced respiratory and osmoregulatory capacity, hyperventilation and ultimately death from suffocation from internal oxygen deficiency. Updated after Marshall et al. (2003) and Mardones et al. (2018).

large amounts of mucus, but may also die after days of exposure. In both cases, the fish seem to die from suffocation (from internal oxygen deficiency) due to mucus production by the fish and/or due to destruction of the gill cells (Andersen et al. 2016, Svendsen et al. 2017; Bergsson et al.2019). Adult fish seem more susceptible to the ichthyotoxins than smaller fish and fish embryos (Binzer et al. 2020). The reason for this is probably that fish embryos and small fish not only rely on oxygen uptake from the gills, but they can take up oxygen via the skin (Binzer et al. 2020). Also, fish with high metabolic rates (e.g. tuna, salmon) seem more susceptible than fish with lower metabolic rates (Svendsen et al. 2017; Bergsson et al. 2019).

Supernatants/filtrates of an ichthyotoxic species are usually less toxic than whole cell cultures (Blossom et al. 2014a; Dorantes-Aranda et al. 2015; Binzer et al. 2020). There may be several reasons for this. First, when the algal cells are separated from the water, the production of these compounds stops. Since many ichthyotoxins are labile and stick to all types of surfaces, the concentration will drop with time causing less effect on the fish. Very few detailed studies of this phenomenon are available, but this has been shown to be the case for *Kl. armiger* and *P. parvum* (Blossom et al. 2014a; Binzer et al. 2020).

Lytic compounds from ichthyotoxic algae may affect other components of the food web, including other algae, micro- and meso-zooplankton as well as benthic invertebrates (Schmidt and Hansen 2001; Tillmann 2004; Binzer et al. 2018; Long et al. 2021). However, the sensitivity of different organisms may be quite different. Some species and groups of organisms are very sensitive, while others are quite robust or not affected by the lytic compounds. Also, some strains that are quite toxic to fish may not, to the same extent, be toxic to for instance other algae (Blossom et al. 2014b). We can only guess as to the reasons for this difference, but it seems likely that cell membranes of fish and algae have different lipid compositions, and that this may be the cause. Also, we now know that ichthyotoxic algae can produce many different congeners of an ichthyotoxin, which can have different toxicities to target cells/organisms. Prymnesin type A has for instance been shown to be six times more toxic than prymnesin B towards the fish gill bioassay (Rasmussen et al. 2016b).

At present, there is no evidence of accumulation of ichthyotoxins in the food chain. In some cases, the ichthyotoxins seem to affect micro and meso-zooplankton even at low cell densities, suggesting that the ingested toxins affect the grazers. This is true, for example, for *P. parvum*, some *Karlodinium* and *Karenia* (Hansen 2005; Tillmann 2004; Vaque et al. 2006; Adolf et al. 2020). In other cases, the grazers will grow even on a mono-diet of the ichthyotoxic alga, as long as the cell density is so low that the grazers are unaffected by toxins released to the surrounding water. This is the case of *Alexandrium*, where toxic strains were ingested at low cell densities and led to high growth and ingestion rates (Hansen 1989; Hansen et al. 1992), and for *K. armiger* where a highly toxic strain was excellent food for the copepod *Acartia tonsa,* when fed low cell densities of the toxic prey (Berge et al. 2012).

Many fish-killing algae are mixoplankton, i.e. combining photosynthesis and phagotrophy. This allows them to compete with often smaller photo-autotrophic algae for nutrients. Ichthyotoxins are secondary metabolites, which primarily affect competitors and grazers, and the effect they cause on fish is often regarded as a side effect. In fact, some fish-killing algae have been shown to use lytic compounds (presumably prymnesins) to immobilize their prey, which otherwise the alga cannot catch. This is the case for *P. parvum* (Tillmann 2003; Skovgaard and Hansen 2003; Medic et al. 2022). Direct predation (called micropredation) as a cause of fish kills has been proposed, in cases where a toxin has not been found or a direct effect of the supernatant cannot be documented (e.g. *Pfiesteria shumwayae*, Vogelbein et al. 2002). While predation on single blood cells, fish gill cells, and micro- and mesoplankton has been clearly documented (Vogelbein et al. 2002), no direct evidence is available suggesting that this is the cause of kills of fish embryos and juvenile fish.

Karlodinium armiger is an example of an ichthyotoxic species that uses micropredation as a means to

kill its larger predators like copepods (Berge et al. 2012) and larvae and embryos of mussels (Binzer et al. 2018). However, it was not possible to demonstrate micropredation on fish larvae (Binzer et al. 2020). Fish gills are protected by mucus that does not allow the alga to attack the intact gills. Rather, the fish gills may be destroyed/lysed by the ichthyotoxins, allowing the toxic alga to detect the cells and opening up them up for predation (Binzer et al. 2020). Recently it was shown that the toxic effects on fish larvae by this alga could be removed almost completely using a resin (HP 20) that removed the extracellular toxins (Binzer et al. 2020).

5.1. Modulation of toxin production and release

The study of the modulation of ichthyotoxins in microalgae is still in its infancy. The cell toxin quota and toxin production rates (toxin quota x division rate (k)) of strains may vary depending on the physico-chemical conditions, such as light, inorganic nutrients, salinity, pH, but also biotic factors such as grazing.

Light has been shown to influence the cell content of prymnesins in *P. parvum* strain (K-0081) by a factor of three, with the highest levels found at high irradiances (Medic et al. 2022). However, not all strains behave the same, and it has been shown that light in *P. parvum* (K-0374) has no influence on prymnesin cell quota (Medic et al. 2022). In both *P. parvum* strains, however, irradiance positively influences the toxin production rate since light positively affects the growth of both strains.

Limitation of inorganic nutrients has early on been shown to increase the apparent toxin contents of ichthyotoxins in *P. parvum* using bioassays (Granéli et al. 2012). A recent study showed that is also the case with prymnesins, which are believed to be the causative ichthyotoxins in *P. parvum* (Anestis et al. 2022). In *Kl. veneficum* karlotoxin cell quotas increase when growth is limited by phosphate (Fu et al. 2010). Karlotoxin production rates normalized to chlorophyll concentrations were not calculated directly in that study, but karlotoxin production rates decreased at low phosphate concentrations.

Prymnesium parvum grows well at salinities in the range 3-30, but blooms usually occur in low salinity waters (Larsen and Bryant 1998; Johnsen et al. 2010; Southhard 2010). Salinity seems to play a role in the toxicity in this species as evaluated from bioassay experiments, but results have not been consistent (Larsen and Bryant 1998). Recent results indicate that prymnesin cell quotas were similar at salinities 5 and 30 in *P. parvum* (Anestis et al. 2022). An algal cell bioassay carried out simultaneously with the measurements of prymnesins indicated that toxicity was higher at low salinity (Anestis et al. 2022). This apparent increase in toxicity in the algal bioassay at low salinity may however be due to a higher susceptibility of the target cells to the low salinity rather than increased toxicity of *P. parvum*. This needs further scrutiny.

Very little is known of the effects of medium pH for the cell toxin quota and production in ichthyotoxic algae. A study of *Kl. armiger* has shown that elevated pH does not affect the cell quota of the ichthyotoxin, karmitoxin. However, karmitoxin production rates have been shown to decrease with low CO_2 concentration due to that the elevated pH affects the growth rate (Svenssen 2019; Binzer et al. 2020). In *Kl. veneficum*, cell karlotoxin quotas increased at low growth rates caused by low CO_2 concentrations (Fu et al. 2010). Karlotoxin production rates normalized to chlorophyll concentrations were not calculated directly, but karlotoxin production rates decreased at low CO_2 concentrations due to low growth rates. The opposite was observed by Müller et al. (2019) when the toxicity was assessed using a fish gill bioassay. A decrease in pH from 8.05 to 7.50 led to a slight decrease in growth rate, but higher *Kl. veneficum* cell density to reach the same LD50 value for gill cell viability.

How biotic factors affect the toxin quota and production rates in ichthyotoxic algae has not received much attention. It is well known that toxin cell quotas and production rates of shellfish toxins (AST and PST) increase when the algae are exposed to copepodamides released by copepods (Grebner et

al. 2019). To what extent cell quotas and production rates of ichthyotoxins are affected is unknown. We also do not know if other algbae and micrograzers (ciliates and phagotrophic dinoflagellates) may elicit increased cell quotas and production rates of ichthyotoxins. Recently, it has been shown that quorum sensing may occur in ichthyotoxic algae. Here a 10-fold increase in prymnesin cell quota was found in a strain of *P. parvum* at high cell densities compared to low cell densities (Anestis 2022). Pathogens may potentially make fish more susceptible to fish-killing algae. In a study by Andersen et al. (2016), experiments were carried out to study if the ichthyotoxic *P. parvum* affects the susceptibility of rainbow trout to viral haemorrhagic septicaemia virus (VHSV). During exposure to sublethal algal densities, the fish increased production of mucus on their gills. When fish were exposed to the algae for 12 h prior to the addition of virus, a marginal decrease in the susceptibility to VHSV was observed compared to fish exposed to VHSV without algae. If virus and algae were added simultaneously, inclusion of the algae increased mortality by 50% compared to fish exposed to virus only, depending on the experimental setup. It was concluded that depending on the local exposure conditions, sublethal *P. parvum* could affect susceptibility of fish to infectious agents such as VHSV.

5.2. Factors affecting the release and degradation of ichthyotoxins

Release rates of ichthyotoxins have not yet been quantified, but it could be expected that release rates correlate with toxin production rates. It is however well established that ichthyotoxins disappear from the water quickly when the algae producing them are removed. Most of these studies were however carried out using cell bioassays and not juvenile or adult fish (e.g Hansen 1989, Hansen et al. 1992; Blossom et al. 2014 a,b). The exact reasons for disappearance of ichthyotoxins are unknown, but irradiance, chemical and bacterial degradation have been suggested. Ichthyotoxins from *P. parvum* have been known for long to be light sensitive (Shilo 1981; Blossom et al. 2014a). Prymnesins are also light sensitive (Taylor et al. 2021) and the released fraction of the toxins does not depend on irradiance, even though prymnesin production rates increased at higher irradiances (Medic et al. 2022). Also, ichthyotoxins, like those from *P. parvum* or the karmitoxins from *Kl. armiger* seem to adsorb to surfaces, like target organisms, and especially plastic and resin materials seem to absorb these toxins rapidly (within hours to days) (Blossom et al. 2014a, Binzer et al. 2020). Different ichthyotoxins behave differently in plastic or glass containers but also in dark and light conditions (Dorantes-Aranda et al. 2015).

5.3. Threshold cell densities for effect of released compounds

Ichthyotoxic algae are not always toxic to target cells and organisms. Below a certain threshold cell density, these algae have no effects. As the density of cells increases, the effects on viability of cells follow a sigmoid “dose: response” curve. This has been shown for unidentified ichthyotoxins using bioassays (e.g. Hansen 1989; Schmidt and Hansen 2001; Granéli and Johansson 2003; Blossom et al. 2014a; Müller et al. 2019), as well as for specific ichthyotoxins like prymnesins, karlo- and karmitoxins (Rasmussen et al. 2017; Binzer et al. 2019). These studies also stress that different target cells/organisms will have different sensitivity to the ichthyotoxins.

Priority topics for research and implementation

- Explore the role of cell density of ichthyotoxic algae for their effects on different target cells under different growth conditions.
- Carry out dose/response experiments with the algae and suspected compounds in ecologically relevant concentrations.
- Explore strains with different toxicities of a given species and verify that there is a relation between toxicity of the strains and the cellular/released suspected causative compound.
- Explore ways to remove the released fractions of the lytic compounds. Ichthyotoxins like prymnesins and karlo/karmitoxins seem to be of a "sticky" nature which can potentially be utilized to remove ichthyotoxins that are released to the water by the algae for better survival of fish during harmful algal blooms.
- Explore the specific modes of action of all ichthyotoxic algae. The possible release of toxins from the algae when the cells get into contact with the gills deserves scrutiny.
- Explore lability/loss of released toxins/agents under different conditions. Ichthyotoxins mainly affect other organisms via release to the water
- Explore role of abiotic factors as a source of variability of cell toxin quota and production.
- Explore role of biotic factors (cell density, co-occurrence of competitors, grazers and pathogens) as elicitors of toxin/agent content and release. Will algae invest more in toxin production when more dense (quorum sensing), and/or when exposed to grazers and pathogens? What is the role of co-factors, like oxygen radicals and PUFAs?

The necessity for bioassays – which ones to select?

Hélène Hégaret and Jorge Mardones

Bioassays are analytical methods which allow one to determine the concentration or potency of a substance, assess its effect on live cells or tissues (*in vitro*), as well as on live organisms (*in vivo*). The use of bioassays also can facilitate their characterization through bio-guided fractionation. In addition, they can be useful to assess the potency of ichthyotoxic compounds or reference materials to be produced.

6.1. Many different bioassays have been used to determine the toxicity of ichthyotoxic algal species or their lytic compounds

A wide diversity of bioassays has been used to determine the potency of ichthyotoxic species, especially those that use different whole organisms such as microalgae, *Artemia,* copepods, shellfish or fish (embryos, larvae or juveniles). Prior to whole fish bioassays, toxicity of microalgal extracts were tested on *Artemia* and erythrocytes of several mammalian species. Meldalh et al. (1994) tested extracts from four ichthyotoxic species, *P. parvum, P. patelliferum, P. polylepis* and *C. leadbeateri* on larvae of the crustacean *Artemia salina* as well as on human erythrocytes, and demonstrated that the *Artemia* bioassay was much more sensitive. Mammalian (human, sheep, rabbit) as well as fish erythrocytes have been used widely to assess hemolytic properties of several microalgae and microalgal extracts. However, these bioassays using cells from live animals or live whole animals cannot be standardized. Furthermore, the lack of homogeneity of technical approaches to compare experimental results has hindered progress in the characterization of ichthyotoxins.

6.2. Bioassays using whole fish, including embryos, larvae, juveniles and/or adults have been especially widely used

The majority of *in vivo* ichthyotoxic bioassays involved only a few fish species, such as rainbow trout (*Oncorhynchus mykiss),* zebrafish *(Danio rerio)* or sheepshead minnows (*Cyprinodon variegatus*). Juvenile rainbow trout were used to evaluate the toxicity of several strains of *P. parvum* (Blossom et al. 2014) by quantifying moribund fish following exposure. Larvae of sheepshead minnows have also often been used as *in vivo* bioassays. This assay was used by Binzer et al. (2020) to assess *Kl. armiger* toxicity. The latter study highlighted that the larval sheepshead minnow bioassay seemed less sensitive than the juvenile rainbow trout bioassay, where they observed slow swimming behavior and loss of balance, followed by upside down floating with respiration as the only movement before death.

Physiological measurements observed and/or assessed on adult and juvenile fishes exposed to ichthyototoxic species or their toxins involve osmoregulatory failure, suffocation, hemolysis, mucus production, cytotoxicity or muscular cramps, with death as endpoint. However, these biological or physiological responses are highly dependent on the health of the target organisms, not enabling standardization. Furthermore, these methods are labor intensive, costly, subject to strict ethical approval processes, thus highlighting the need to develop cell-based bioassays. Additionally, while the use of whole organisms, including juvenile or adult fishes, provides robust and ecologically relevant

results, they require special rearing facilities, a large volume of algae and often animal ethics approvals.

6.3. *In vitro* assays have also been developed targeting hemocytic or allelopathic effects or effects on embryos, hepatocytes or gill cell lines

In vitro models have allowed direct assessment of specific functions in many areas of life sciences, with higher control of the conditions of assays and, decreasing variability of responses due to unavoidable environmental stress. Furthermore, only small quantities of samples are usually necessary, with shorter times of exposure compared to *in vivo* whole organisms assays to detect response (Wernersson et al. 2015). *In vitro* cell-based bioassays appear to be much more cost-effective, manageable to high throughput, and allow for short time testing. Moreover, depending on the target cell, they can represent the primary targets of chemically induced effects, without needing to use the whole animal. Several cell-based *in-vitro* bioassays have been targeted to investigate ichthyotoxic properties, involving cell line bioassays, such as mammalian or fish cell lines or microalgal cells from culture collections. Cell lines have offered a methodological alternative to the use of whole organisms for testing marine toxicants with experimental advantages that include faster results, simplicity, a reduction in number of experimental fish, cost-effectiveness and specificity. The use of *in vitro* assays has increased over the past decades for ethical reasons to comply with increasing regulations on animal experimentation.

6.4. *In*-vitro allelopathic assay on microalgal cultures

Clonal microalgal strains, available in culture collections, to assess the effects of ichthyotoxins have been used extensively. They allow for a better standardization of results, as these known strains, if cultured under the same conditions, can provide the same physiological response, thus enabling the comparison of allelopathic potential of microalgal species. Allelopathic bioassays assess the allelopathic potential of the microalgae but not directly their ichthyotoxicity. Most assessments of were originally performed measuring cell mortality assessed by light microscopy (Tillmann and John 2002; Tillman et al. 2007, 2008). These measures of algal mortality have allowed to provide LC50 for allelopathic microalgal strains and species. However, light microscopic analyses are time consuming and alternative methods have also been developed, such as measurement of chlorophyll fluorescence using spectrofluorometry (Blossom et al. 2014), or measurement of maximum quantum yield (Fv/Fm) by pulse-amplitude-modulation (PAM) fluorometry (Tillmann et al. 2007; Lelong et al. 2011). The latter method was adapted using the *Chaetoceros muelleri* strain CCAP1010-3, a diatom easy to culture (Long et al. 2018). This *Chaetoceros* QY bioassay requires very low amount of allelopathic mixture (microalgal extracts - filtrate or lysate), is simple and quick to perform, providing semi-quantitative results, and requires only a PAM fluorometer, which can be transportable for field work. This bioassay appears a good candidate for systematic and standardized allelopathic measurements of lytic microalgal species.

These assays are based on the assumption however that ichthyotoxins and lysins are also the allelopathic compounds measured. Even though it is possible to confirm this hypothesis for known ichthyotoxins, for most ichthyotoxic microalgal species, for which the compounds have not been described yet, this assumption still needs to be verified. Several studies seem to validate this, as reported for *M. polykrikoides* (Tang and Gobler 2010) or *A. minutum* (Long et al. 2021). However, Blossom et al. (2014), using five strains of *P. parvum* showed that allelopathy towards the microalga *Teleaulax acuta* did not seem to be correlated to ichthyotoxicity towards juvenile rainbow trout. The latter study highlighted the higher susceptibility of juvenile fish than of the microalgae to *P. parvum.* The use of allelopathic

assays in order to assess ichthyotoxicity thus requires the verification that allelopathic measurement indeed provides a measurement of the ichthyotoxic compounds.

6.5. Cell line bioassays to test fish-killing toxins

Cell lines of different origin have been used to test the mode of action of a wide diverse of toxic metabolites derived from FKA. For instance, Vero (African green monkey kidney) and mouse neuroblastoma cell lines have been used for testing cytotoxic compounds produced by *Alexandrium tamarense* (Katsuo et al. 2007). The Vero cell line has also been used for the assessment of the toxic activity of the dinoflagellates *A. affine, A. fraterculus, A. tamiyavanichii*, and *Heterocapsa circularisquama*. A major difficulty of working with mammalian cell lines is that these types of cells require incubation and maintenance at 37 °C in a 5% CO_2 atmosphere. By contrast, fish cell lines can be cultured between 18 and 22 °C, which is compatible with the temperature of growth of a wide range of toxic microalgae.

6.6. The RTgill-W1 cell line assay seems most promising, showing high sensitivity and has been submitted for ISO-OECD certification

The application of a standardized and highly sensitive and reproducible rainbow trout RTgill-W1 cell line assay has provided an opportunity to study both cytotoxicity and biotransformation of marine toxins at the branchial level in much more detail than is possible *in vivo* (Dorantes-Aranda et al. 2011). The fact that the mode of action of most ichthyotoxins has been shown to be related to the disruption of the cell membrane by specific binding to, for instance, cholesterol, thus creating pores in the membrane (Place et al. 2009), makes the epithelial fish RTgill-W1 cell line an excellent candidate for testing these types of algal toxins. Mooney et al. (2011) demonstrated that the RTgill-W1 cell line was more sensitive than the larvae of sheepshead minnow (*Cyprinodon variegans*) upon exposure to fatty acids produced by toxic dinoflagellates. Rasmussen et al. (2017) showed that karmitoxin produced by the mixotrophic dinoflagellate *Kl. armiger* had a potent cytotoxic activity at a nanomolar concentration using the RTgill-W1 bioassay. Thus, the use of this highly sensitive bioassay has allowed a rapid screening of samples, and when performed under controlled conditions, it allows comparative studies of a wide range of ichthyotoxic algae or their lysins among different species and strains (Dorantes-Aranda et al. 2015; Fig 6.1). However, there are still several steps until its complete standardization. A main constraint of using the RTgill-W1 to test the ichthyotoxicity of harmful marine microalgae is that this cell line is not sufficiently saline-tolerant and cannot be directly exposed to toxic microalgae in seawater for more than 3h (Dorantes-Aranda et al. 2011). This problem could be solved using Transwell-type membrane chambers to achieve cell survival for at least 3h with the upper compartment holding the algae in seawater and the lower compartment containing L-15/ex medium. However, membrane supports within microplate inserts are expensive and prevent its use on a routine basis. An alternative approach using red sea bream (*Pagrus major*) gill cells, a fully marine fish (oceanodromous), makes it unnecessary to acclimate the cells to seawater, thereby making it possible to directly expose the cells to harmful marine algae using conventional plates (Ohkubo et al. 2017). In terms of toxicity end points, the RTgill-W1 assay is evaluated photometrically by measuring a fluorescence dye indicating metabolic gill cell activity (Dorantes-Aranda et al. 2011).

For standardization purposes, a recent published ISO-21115:2019 procedure suggests the use of two additional endpoints for measuring the integrity of the cell membrane and integrity of the lysosomal membrane using CFDA-AM and neutral red fluorescent indicator dyes, respectively. Previous studies using the RTgill W-1 gill assay only assessed the effect of "artificial lab conditions", bypassing "realistic environmental scenarios." Mixtures of compounds (a real scenario for FKA toxins monitoring) have received much less attention, and the effect of physico-chemical factors on the toxicity of mixtures of

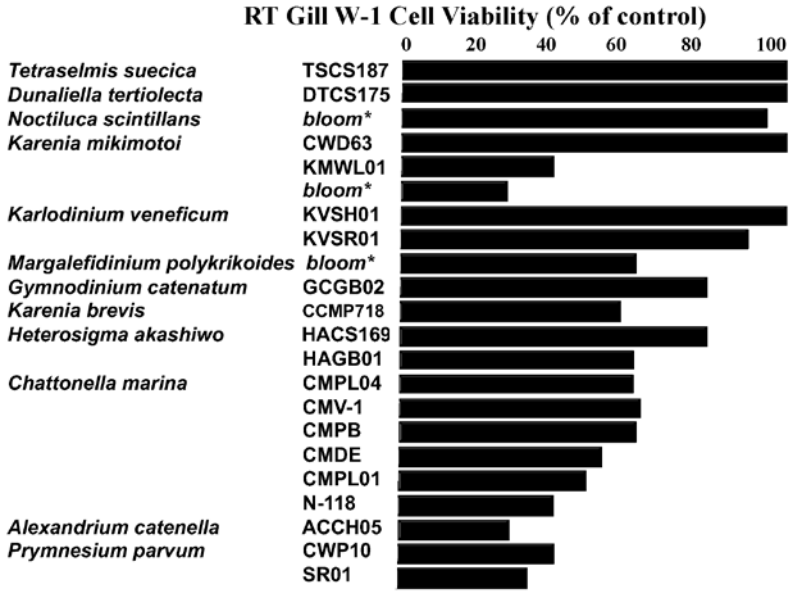

Figure 6.1. Performance of the RTgill W-1 bioassay within a single laboratory (University of Tasmania) on freshly lysed algal cultures, arranged from least to most ichthyotoxic. Note the inclusion of natural bloom samples (asterisks) of *Noctiluca scintillans* (Tasmania), *Karenia mikimotoi* (South Australia) and *Margalefidinium polykrikoides* (Korea). Updated from data in Dorantes-Aranda et al. (2015). Considerable strain variability is evident for *Karenia mikimotoi*, *Heterosigma akashiwo*, and *Chattonella marina*.

FKTs even less attention. Addressing these key questions might potentially allow the final determination of acute fish toxicity by FKA in chemically complex natural marine waters.

6.7. There exists neither a standardized bioassay, nor a methodology to systematically assess toxicity of ichthyotoxic/lytic algae and their toxins

So far, no standardized bioassays have been developed to assess ichthyotoxicity. Both the RT-gill W1 and the *Chaetoceros* QY bioassays are two potential complementary candidates for standardized bioassays. The RT-gill W1 bioassay is available as "standardized" cell-lines, allows the testing of both algae and their extracts and is already widely used in testing chemical pollutants of aquatic ecosystems. Moreover, gills are the main tissue in direct contact with ichthyotoxins. As a complement, the *Chaetoceros* QY bioassays provides for a faster, simpler assay, which can be easily performed in the field for a first assessment of toxicity. The comparison between both bioassays has not been performed for an array of lysins or ichthyotoxic microalgae.

Although the RTgill-W1 assay has proven to be the most promising standardized method for the quantification a characterization of ichthyotoxins, it must be kept in mind that the response of this epithelial gill cell line does not necessarily represent the complex response of the whole organism. Similar questions arise for the *Chaetoceros* QY bioassays, assessing allelopathy effects rather than ichthyotoxicity. Moreover, there are still key questions that remain to be solved in order to use this assay in routine monitoring, such as: 1) How to discriminate the effect of different toxins (if more than one present) in a blind seawater sample?; 2) Can two or more toxins act in synergy against the target cells?; 3) Do water samples from different environments (i.e., estuarine, oceanic) produce a differing cell response based on osmotic processes?; 4) Can physico-chemical factors (temperature, pH, storage time and light exposure) change the toxic potency during sample transport?; and 5) How should cell bioassay responses (gill or microalgal cells) be interpreted to determine critical toxic levels for whole fish?

Another important issue when screening unknown ichthyotoxins are the methodological aspects as described by Long et al. (2021): the culture phase or health status at which microalgae are sampled,

the procedure of collection and preservation of the extracts or the potential interference of laboratory materials (i.e., culture flasks, microplates, pipette tips). Adsorption of ichthyotoxins by laboratory materials has been claimed to occur due to hydrophobic interactions with plastic surfaces, with porosity thickness and roughness interacting as co-factors (Hyenstrand et al. 2001). For instance, Dorantes-Aranda et al. (2015) using the RTgill-W1 assay determined that glass materials were more suitable for testing brevetoxins and methanol extracts of the haptophyte *P. parvum*. By contrast, polystyrene materials showed better results with extracts from the dinoflagellates *Amphidinium carterae, K. mikimotoi,* and *K. veneficum*, and with karlotoxins. For assessing potency of *A. minutum*, the use of specific filters (acetate cellulose or asymmetric polyethersulfone) was recommended (Long et al. 2018). This highlights the need to standardize methodologies and protocols, which must be adapted for each ichthyotoxic microalgae and ichthyotoxin.

6.8. It is important to develop an *in-situ* bioassay allowing on-site monitoring for stakeholders and scientific community

Being able to assess toxicity of ichthyotoxic blooms in the field, as the bloom occurs appears as critical in fishery management. The development of an *in-situ* bioassay would allow on-site quantification of the ichthyotoxic potential of a bloom, thus enabling for specific management/aquaculture strategies to avoid or limit mass mortalities of fish and shellfish. So far, no specific *in-situ* bioassays have been developed. The fish-gill bioassay could be a good candidate, but remains relatively complicated to implement in the field or on boat, but has been successfully applied to unpreserved water samples transported to a shore-based lab within 12-24 h (Seger et al. 2017). A promising alternative could be the use of the diatom *Chaetoceros muelleri* as a target species for detecting ichthyotoxic activity throughout the use of pulse amplitude modulation (PAM) fluorometry (Long et al. 2018), since the PAM fluorometer can be carried in the field and requires low amounts of material.

Priority topics for research and implementation

- Develop a sensitive bioassay to detect a wide range of lytic/ichthyotoxic algae or their lysins within the frame of *Replacement, Reduction and Refinement*.
- Develop a standardized bioassay as a proxy for acute fish toxicity, based on their different mode of actions and to replace the use of whole live fish.
- Aim for low-volume bioassays. Fish-gill bioassays and *Chaetoceros* QY bioassays both appear as good candidates.
- Validate standardized bioassay with international laboratory inter-calibration.
- Develop an *in situ* bioassay for on-site monitoring available to stakeholders, scientific community. The fish-gill bioassay RT-gill W-1 appears the most promising one, but may not always be implementable in the field.
- Need for manual/guide of good practices when handling ichthyotoxic/lytic algae and lysins.
- Need for international training courses to properly use standardized bioassays.

Broader ecosystem impacts

Lincoln MacKenzie

Figure 7.1. Mortality of marine organisms off the South African west coast, caused by entrapment and subsequent decay of a massive non-toxic dinoflagellate bloom (*Tripos furca, Prorocentrum micans*). Oxygen concentrations decreased to <0.5ppm and anaerobic bacteria generated hydrogen sulphide concentrations up to 50µmol/L. This caused the sea to turn black. Some 60 tons of lobster and 1500 tons of fish, comprising 50 species, washed ashore. Photo: the Argus newspaper.

Marine mass mortality events are common worldwide, although in many cases it may be difficult to unambiguously attribute toxic algal blooms as the cause (Landsberg 2002). Fish-killing microalgal blooms may or may not have significant wider ecosystem effects. The most important determinant of the nature and scale of such effects is the species of microalgae involved, bloom intensity, timing, duration, spatial extent, hydrography, salinity, temperature and other environmental factors. Some events are infrequent, spatially limited and short lived, while others are extensive, of long duration, and frequently cause massive marine fauna kills (e.g. *K. brevis* blooms in the Gulf of Mexico; Pierce and Henry 2009).

Fish-killing blooms can play an important role in the periodic reshaping of the structure of benthic and pelagic communities and cause biodiversity losses that drive long term ecosystem change. However, with a few notable exceptions (e.g. Wear and Gardner 2001, Gjøsæter et al. 2000, Jurgens et al. 2015), there have been few studies on the long-term consequences of toxic algal bloom having induced multiple species mass mortalities. Harmful microalgae can interact in complex ways with benthic communities on rocky reefs. In several instances HABs have been implicated in altering the dynamic equilibrium between macrophytes and their grazers (e.g. kelp forests vs urchin barrens). Shears and

Ross (2010) investigated the interactive effects of fishing and HABs on a predator-sea-urchin-macroalgae trophic cascade. They found that macroalgal recovery from urchin grazing was enhanced by the debilitating effects of an *Ostreopsis siamensis* benthic dinoflagellate bloom on urchins that made them more susceptible to fish predation. The potential for chronic or acute exposure of harmful algae to fish and other biota to increase their susceptibility to or even act as vectors for infectious disease has not been well studied.

7.1. Physical damage to fish gills/ deoxygenation events

Microalgae that cause mechanical damage to gill tissues (such as diatoms and silicoflagellates) are not usually associated with wider ecosystem effects although they may exacerbate the impact of other environmental stressors such as abnormally high temperatures (Roberts et al. 2019). There exist numerous reports of deoxygenation events caused by the collapse of high-biomass phytoplankton blooms (e.g. Taylor et al. 1985; Pitcher and Probyn 2016; Cockcroft et al. 2000) having devastating impacts on benthic communities (Pitcher and Jacinto 2020). Wild finfish usually avoid such situations and it is believed they are rarely affected. Such high biomass HAB phenomena are not covered here.

7.2. Raphidophyte and dictyochophyte blooms

Fish-killing raphidophytes (e.g. *Heterosigma akashiwo, Chattonella marina*) are well known to have lethal effects on captive finfish (Lum et al. 2021). While the precise mechanism is still debated (Grogan et al. 2015, Dorantes-Aranda et al. 2015), intimate contact of algal cells with the gills of the fish is clearly required. There are some accounts during blooms of these species of harmful effects on other biota (salmon, molluscs, crustaceans, tunicates) (Hershberger et al. 1997; Barraza Guardado et al. 2002) but usually mortalities of fish in sea cages take place without any obvious signs of more extensive ecological effects (MacKenzie 1991). There are two described species of *Pseudochattonella* (Dictyochophyceae) that have become globally recognised as fish-killing algae with potent effects on sea-cage aquaculture (Mackenzie et al. 2011; Ekford-Soper and Daugberg 2015). Elucidation of the mechanism by which *Pseudochattonella* is lethal to fish is unresolved but clearly involves damage to fish gills through contact with live cells. There exists one report of severe impacts on wild sea-trout populations in Denmark (Andresen et al. 2015) but otherwise, there is little evidence of mass mortalities of other flora and fauna during *Pseudochattonella* blooms.

7.3. Toxin-producing blooms

Where intra-cellular or excreted toxins are clearly implicated (mainly from dinoflagellates and haptophytes) the impact on wild and captive fish is frequently associated with blooms that cause multiple species mass mortalities of marine flora and fauna.

7.3.1. *Karenia* species

One of the most thoroughly documented studies of the long-term ecosystem effects of a *Karenia* bloom was reported by Gardener and Wear (2001) and Wear and Gardener (2006). This bloom of *K. brevisulcata* (Chang 1999) occurred in Wellington Harbour, New Zealand in the summer of 1989 resulting in mass mortalities of zooplankton, pelagic and demersal fish and benthic invertebrates including polychaetes, gastropod and bivalve molluscs, echinoderms and crustaceans. Dinoflagellate cell numbers exceeded 3,000,000 cells/L. Large quantities of sea-foam were generated during the peak and collapse of the bloom which was associated with an outbreak of respiratory irritation syndrome. A complex suite of novel lipid and water-soluble toxins were characterised from cultures of *K. brevisulcata* (Hol-

land et al. 2012). Previous research on the flora and fauna at a network of intertidal and subtidal sites in Wellington Harbour provided excellent baseline data to enable evaluation of the initial impact of the bloom and recovery of the ecosystem over subsequent years. The bloom had a profound effect on the whole inlet basin (82 km^2), from the deepest sub-tidal to highest inter-tidal habitats. Deeper burrowing in-fauna survived better than shallow burrowing animals and the low energy silty environment of the central harbor basin was impacted more severely than shallower areas subject to strong tidal flows and wind-induced turbulence. The bloom affected an aquaculture research facility on the shores of the harbour. Large numbers of cultured turbot and seahorses were killed and brine shrimp and algal cultures were destroyed via filtered sea water entering the hatchery's seawater reticulation system. Reexamination of benthic fauna after 8 and 12 months showed rapid rates of recovery and re-colonization of soft bottom habitats (Kroger et al. 2006) but recovery was site-specific. Complete recovery to the pre-disturbance situation was estimated to take 4-5 years. Wear and Gardener (2006) concluded that the *K. brevisulcata* bloom did not result in profound long-term modification of benthic biological communities.

Karenia mikimotoi blooms have often been identified globally as the cause of extensive mass mortalities of a wide variety of benthic and pelagic fauna (e.g. Silke et al 2005; Davidson et al. 2009). Affected species have included finfish (blennies, gobies, conger eel, butterfish, flat fish and cultured cod), heart urchins, brittle stars, lugworms, cockles, mussels, scallops, whelks, penis worms, heart urchins, edible sea urchins, sea cucumbers and lobster. A survey of benthic communities in Killary Harbour was carried out after the bloom to assess differences in the benthic community at the same sites 2 years previously. Differences were due to the removal or reduction of sensitive taxa such as sea urchins which appear particularly susceptible to the effects of fish killing *Karenia* blooms (e.g. Iwataki et al. 2021).

Mass mortalities of finfish associated with blooms of brevetoxin producing *K. brevis* in the Gulf of Mexico have been observed for centuries, and continue to occur in this region. Other ecological (e.g. brevetoxicosis of sea birds, turtles and marine mammals), human health (e.g. respiratory irritation syndrome) and economic (fisheries and aquaculture disruption) impacts have also been reported in recent years (Fauquier et al. 2013; Foley et al. 2019; Flewelling et al. 2005; Abraham et al. 2021). More subtle and less easily observed ecological consequences probably also occur. These may include effects such as recruitment failures (Summerson and Peterson 1990) or affect bivalve larval development (Leverone et al. 2006) and restructuring of pelagic communities through changes in species diversity, dominance and food web structure (DiLeone and Aisworth 2019; Berens-McCabe et al. 2021), ultimately affecting the prey assemblages of apex predators such as dolphins. The underlying reason why *Karenia* spp. produce such an array of toxic compounds has been partially illuminated by Errera and Campbell (2011), who demonstrated that osmotic shock triggers toxin production by *K. brevis.* Perhaps other bioactive compounds (e.g. gymnodimine) produced by other *Karenia* spp. have similar osmoregulatory functions.

In addition to *K. brevis* and *K. mikimotoi* there are several other naked dinoflagellate species that have been associated with toxic effects causing mass mortality of marine fauna including *K. selliformis* (Iwataki et al. 2021, Mardones et al. 2021, Orlova et al. 2022), *M. polykrikoides* (Lee et al. 2013) and *Karlodinium* spp. (Lin et al. 2014).

7.3.2. *Alexandrium* blooms

Blooms of *Alexandrium* spp. are primarily regarded as mediators of Paralytic Shellfish Toxin (PST) contamination of filter feeding shellfish, although they are also well known as being responsible for mass mortalities in molluscan, fish, mammal, and seabird populations (Shumway 1990, Shumway et al. 2003, Starr et al. 2017, Ben-Gigirey et al. 2021) including cultured fish in sea cages (Fuentes et al.

2008). PST-producing blooms have also been identified as a major cause of endangered sea turtle mortalities (Amay et al. 2018) and there are numerous reports of PST-producing blooms impacting the survival of early life cycle stages of bivalves (e.g. Mu and Li 2013). Deaths of marine fauna may occur either through direct ingestion or food chain transmission of PSTs, or other mechanisms such as the synergistic effects of reactive oxygen species and PUFAs (Mardones et al. 2015, 2018), or as yet uncharacterised bioactive extra-cellular compounds on gill tissues (Castrec et al. 2018, Long et al. 2021).

7.3.3. Yessotoxins

Yessotoxins (YTXs), produced by at least two species of dinoflagellate (Stake et al. 1997; Rhodes et al. 2006), were first identified as a toxic contaminant of filter-feeding bivalves. There are no known accounts of YTX being toxic to captive finfish, but this toxin has come to some prominence in recent years as the cause of mass mortalities of farmed and wild shellfish (especially abalone) and echinoderms (Pitcher et al. 2019; King et al. 2021) with profound flow on effects on community structure and ecosystem function (Jurgens et al. 2015).

7.3.4. Haptophyte blooms

Gjøsaeter et al. (2000) provided a comprehensive overview of the long-term ecosystem consequences of a *P. polylepis* bloom which impacted coastal and offshore waters of Scandinavia in 1998. The bloom was extensive (75,000 km^2), with exceptionally high cell numbers (50-100 $x10^6$ cells/litre) concentrated in thin layers near the pycnocline. Farmed and wild fish, molluscs, echinoderms, ascidians, cnidarians, sponges and red algae were severely affected by the bloom. Juvenile cod experienced high mortalities in June-November 1988 and this year class was subsequently reduced. Older fish were less affected probably because they could move from bloom affected waters. Fish exposed to the bloom suffered gill damage and subsequent respiratory failure although *P. polylepis* was less harmful to fish at salinities of 20 psu or less. Intertidal carnivorous welks were most affected, but mussels and barnacles much less. Subtidal sea stars, urchins, ascidians and bivalves were almost completely eliminated in some areas.

Despite the widespread devastation of marine communities caused by the bloom, comparison of nearshore fish communities 3-6 years before and after the bloom showed few differences and there were negligible long-term effects. Benthic invertebrate communities were strongly affected in the short term but recovered surprisingly fast and populations of most soft bottom organisms recovered within months, and after one year, little evidence of damage was noticed. Below the pycnocline, most species survived, and after the bloom dissipated, recolonization of shallower depths was rapid. On hard bottom substrate, there was heavy mortality of kelp and *Asterias* star-fish predators, which subsequently resulted in colonisation of the vacant habitat by mussels. However, within two years the star-fish population restablished itself and largely removed the mussels, thus providing the opportunity for the return of a brown algal dominated community and the revival of the original benthic community within five years.

Conclusions

Wider ecosystem effects are common for fish-killing blooms where intracellular or excreted toxins are primarily responsible (e.g. *Karenia* spp. and *Prymnesium*/*Chrysochromulina* spp. blooms). Ecological effects appear less common with blooms of raphidophytes (e.g. *Heterosigma)* and dictyochophytes (e.g. *Pseudochattonella*) that require intimate cellular contact for harmful effects. Many marine mass mortality events attributable to phytoplankton may go unnoticed or undocumented. Transient environmental factors during blooms (e.g. salinity, light, temperature, nutrients) may moderate or exacerbate

the wider ecological effects of fish-killing blooms. The consequences of effects of extra cellular toxins on micro-planktonic organisms (e.g. mollusc larvae) may be a hidden cause of recruitment failures within benthic communities. The most thorough, long-term studies on ecosystem impacts of fish killing blooms suggest that although localised effects may be catastrophic, recovery can be rapid, and long-term effects are negligible. The ecological effects of fish-killing blooms may play important roles in reshaping planktonic and benthic community structure and function. On the one hand, they can cause significant negative environmental effects and the destruction of valuable fisheries resources. On the other hand, they may be analogous to wild fires, periodically thinning out populations, maintaining species diversity and enhancing resilience.

Priority topics for research and implementation

- DInvestigate ecosystem effects by known toxin producing organisms (e.g. *Karenia* spp.) versus fish-killing species where the mechanism is as yet unknown (e.g. raphidophytes).
- Can impacts on farmed fish be used as an indicator also of wild fish susceptibility to HABs?
- Explore the role of ichthyotoxic blooms in initiating tropic cascades due to relative changes in the populations of predators and prey with subsequent changes in ecosystem structure and nutrient cycling.
- Improve understanding of the causes of marine mass mortality events associated with algal blooms as these occur against a background of ecosystem disturbances and global climate related changes in the marine environment.
- Why are abalone, sea urchins and some benthic invertebrates so vulnerable to yessotoxin, gymnodimine, etc., producing HABs?

Ecophysiology of fish-killing algae and role of climate change

Gustaaf Hallegraeff

Climate change can affect HABs through warming, elevated CO_2 (both stimulating photosynthesis and driving ocean acidification), increased stratification, changes in light and nutrient availability, heavy rainfall and land runoff, and impacts on grazers (Hallegraeff 2010, Wells et al. 2021). Over the past 15 years, the science of how HABs respond to climate has progressed from single-factor to multistressor single-species laboratory and multi-species mesocosm manipulation experiments to predictive modeling. To progress HAB climate science, a 'best practices' manual on experimental protocols, use of biological reference organisms, climate 'hot-spot' observer sites and analysis of plankton time-series is now available (GlobalHAB 2021). Developing HAB predictive capability has been slowed by contradictory information on species responses and especially strain-specific responses, lack of insights into evolutionary adaptation; of how HABs interact with the broader phytoplankton and zooplankton grazer communities; and scarcity of sustained (>30 years) biological data streams. Not only do different HAB species respond differently, increasingly sophisticated climate models have pointed out how different ocean regions will change at different rates in temperature, oxygen, nutrients, and ocean acidification (Bopp et al. 2013). We also can learn from extreme climate events such as the 2014-2016 marine heatwave and the 2016-2017 El Niño, which unpredictably triggered different HAB functional groups (dinoflagellate *Alexandrium,* diatom *Pseudo-nitzschia,* dictyochophyte flagellate *Pseudochattonella*) in different parts of the same Pacific basin (Trainer et al. 2019). Emerging HAB climate responses

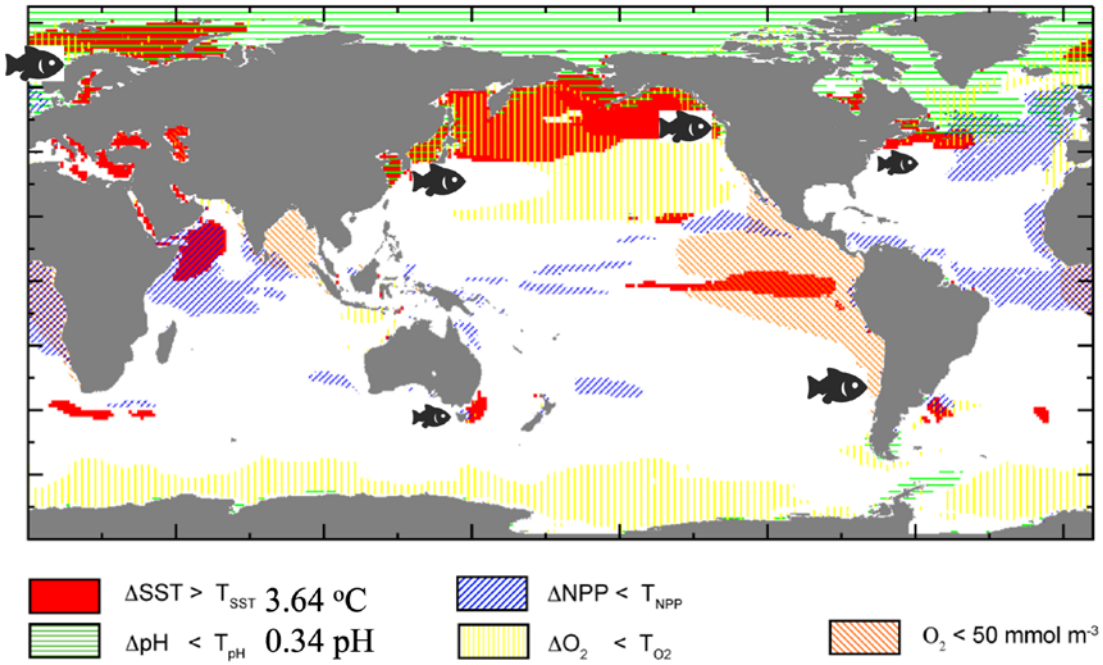

Figure 8.1. Different ocean regions change at different rates. Global hotspots in terms of temperature change (red), ocean acidification (green), nutrient alterations (blue) and lowered oxygenation (yellow and orange) are indicated. Modified after Bopp et al. (2013), based on RCP8.5 model predictions comparing 2090s with the 1990s. Fish symbols indicate the sites of major finfish kills in the past decades.

Table 8.1. Summary of existing knowledge on environmental drivers of fish killing HABs

HAB species	Temperature	Stratification	Increased pCO_2	Nutrients	Ichthyo toxicity	Expected trend with Climate change	References
Alexandrium catenella (= *fundyense*)	Cold-water preference	++	Increased growth; increased toxin (PST, strain-specific)		Lytic compounds	Range expansion Chile; change of temporal bloom window in Puget Sound	Fu et al. 2012; Hattenrath-Lehmann et al. 2015(NPB8, CCMP2304 strains);Gobler et al. 2017
Margalefidinium spp. (*Cochlodinium spp.*)	In warm temperature: Increased growth, but less toxicity	+ (migration)			Toxicity	Increased biomass; biogeographic expansion	Griffith et al. 2019; Griffith & Gobler 2016
Karenia brevis *K. mikimotoi*	In warm temperature: Increased growth	++	Increases growth; No effect on toxicity	P deficiency drives brevetoxins	Triggered by osmotic shock	Increased biomass	Hardison et al. 2014; Errera et al. 2014 (CCFWC268/SP1 strain); Wang et al. 2019. DP-C32
K. selliformis	Cold-water preference		pH tolerant				Vellojin et al. 2023
Karlodinium veneficum	In warm temperature: Increased growth	+	Increases toxicity	P deficiency drives toxicity	Role of prey		Fu et al. 2010; Müller et al. 2019; (KVSR01 strain)
Heterosigma spp. *Chattonella* spp.	In warm temperature: Increased growth	++ (migration)	High pH: Increased growth		Variable Toxicity. Reduced ROS at pH>9. Role of predators		Fu et al. 2007; Giesler et al. 2023
Pseudo-chattonella spp. *Vicicitus globosus*		++	Mesocosm bloom at 800ppm		Variable toxicity; only when mucocysts are produced. More toxic at 25 than 35 ppt	Range expansion, including Chile	Anderson 2015; Mardones et al. 2019; Trainer et al. 2019; Riebesell et al. 2018
Prymnesium parvum				N and P deficiency drives toxicity	Increased at lower salinity, high light and higher biomass; role of prey		Larsen & Bryant 1998; Medic et al. 2022

Note: "++" means strong impact, "+" means positive impact

include: range expansion of warm-water at the expense of cold-water species; changes in abundance and seasonal bloom window; and increased cellular toxin content of key HAB species.

Table 8.1 summarises existing knowledge on the ecophysiology of key fish-killing HAB taxa. Some species respond to nutrients as the key driver (*Karlodinium, Prymnesium, Chattonella*), and most HABs show higher growth rates at higher temperatures (with exception of the cold-water *Alexandrium catenella* and *Karenia selliformis*). Being flagellates, nearly all are favoured by increased water column stratification. *Heterosigma* and possibly *Pseudochattonella* also respond to salinity stratification (Sandoval-Sanhueza et al. 2022). Lower salinity stimulates cytotoxicity by both *Heterosigma* and *Pseudochattonella* (Ikeda et al. 2016, Mardones et al. 2019). High biomass does not necessarily mean high ichthyotoxicity, however; e.g. phosphorus deficiency appears to drive toxicity of *K. brevis* (Hardison et al. 2014), *Kl. veneficum* and *P. parvum*. While increased pCO_2 drives increased *K. brevis* biomass in one study, it does not affect brevetoxin cell quota (Errera et al. 2014), and similarly does not influence ichthyotoxicity by *K. mikimotoi* (Wang et al. ,2019). While increased pCO_2 did drive increase PST toxicity in some *Alexandrium* strains, but not others (Tatters et al. 2013, Hattenrath-Lehmann et al. 2014), we have no knowledge yet on the identity of the lytic compounds produced by *Alexandrium*, let alone its ecophysiological drivers. For *Chattonella marina*, ichthyotoxicity again is strain-specific, related to high light intensity and possibly Fe-deficiency generating ROS, and being most toxic when dark-adapted cultures are suddenly exposed to light (Dorantes-Aranda et al. 2013). For species where cell-lysis is critical for ichthyotoxicity, the most fragile species (e.g. *Pseudochattonella*) or most fragile strains (e.g. *Chattonella*) pose the greatest risk, notably under senescent bloom conditions or osmotic shock in estuaries or frontal systems. The strong vertical migration capabilities of *Pseudochattonella,*

Alexandrium, Margalefidinium, Heterosigma allows them to generate (difficult to detect) dense subsurface layers, e.g. in Chile under cold-brackish conditions for *Heterosigma* and hot salty conditions for *Pseudochattonella* (Sandoval-Sanhueza et al. 2022).

Several attempts have been made to review the impact of aquaculture effluents in driving HABs (Gowen and Bradbury 1987, Forrest et al. 2007, Brown et al. 2020), but unlike the demonstrable impact of fish farms on sessile benthic invertebrate communities, a lasting impact on transient plankton communities has so far been difficult to prove. While some linkages between eutrophication and high-biomass blooms have been demonstrated, these links are not consistent (Gowen et al. 2012). While some studies have demonstrated an increase in chlorophyll concentration (as an index of algal biomass) in proximity to fish farming (Honkanen and Helminen 2000, Nordvarg and Håkanson 2002, Modica et al. 2006), others have not (Husa et al. 2014, Pitta et al 2009). In their reviews of the evidence for and against the fish farm nutrient - HAB hypothesis, neither Rydberg (2003) nor Smayda (2006) could find evidence for a causal link.

However, the global increase in aquaculture production drives increased HAB observations and can bring to light new problem species (Hallegraeff et al. 2021). The dinoflagellate *Karlodinium australe* thus never caused any problems in its Australian lagoon-type locality, but in 2014 killed 50,000 caged fish in Malaysia and is now also known from Japan and the Philippines. The incidence of fish-killing HAB species is not an accurate predictor of economic losses. For example, *Heterosigma* blooms occur both on the west and east coast of North America, but fish mortalities are mostly confined to the west coast. In the wild, finfish can swim away from bloom areas, hence aquaculture finfish mortality is partly a human-generated problem.

Priority topics for research and implementation

- A major gap in our knowledge is how lytic compounds will respond to changes in temperature, pCO_2 and nutrient (N, P, Fe) availability.
- Climate models can help in predicting range expansions or changes in seasonal bloom window, but model predictions or oversimplified laboratory simulations do not always match reality.
- Climate change is adding a new level of uncertainty to seafood safety and HAB monitoring programs, calling for more investment in global observation systems, improved ocean sensor capabilities and integrated data management, and for fish farms, in particular, the availability of rapid-response mitigation options.

Prevention, control and mitigation

Donald Anderson and J.E. Jack Rensel

Mariculture fish farms include highly diverse types, sizes, locations, and degree of sophistication ranging from large-scale, mechanized farms operated by large multinational companies to small-scale, family-operated farms. The largest concentrations of large-scale, mariculture net pen operations are in Norway and Chile, but they also occur in North America (primarily Canada, Panama and Mexico), Scotland, Ireland, Iceland, Sweden, Finland, Spain, some other European and Mediterranean Sea coastal zones, several countries in the Middle East, India, Australia and several countries in Southeast Asia, South Korea and Japan. Small-scale mariculture farms predominate in Southeast Asia and India, but the quantity of these farms, and their total production has grown dramatically in the past few decades. Farmed seafood remains an important cultural and food supply component of many countries. South Korean and Japanese marine mariculture farms vary from small-to-medium scale and may involve fish and concurrent seaweed and shellfish production. Over 50% of the world's fish are now provided by fish culture.

When HABs are involved in large-scale fish farming losses, newspaper and trade journals may report generalities such as numerical and financial losses. The industry suffers from a lack of data sharing, although in some regions, monitoring consortia function to collect data and share them among participants. Rarely are data made public regarding the efficacy of mitigation efforts except for general descriptions in trade journals. In rare cases, researchers are allowed to assess and report mitigation efficacy, but the large size of many facilities creates difficulties to accurately quantify results throughout an array of pens.

Fish farming in net pens has been controversial in some regions and well accepted in others. Norway is the largest producer of farmed salmon and trout and employs many residents but was subject to a national resource rent increase of 40% of business income in 2023 for larger marine fish farms. Revocation of net pen permits by politically elected or appointed government ministers in Washington State and British Columbia, Canada have recently occurred in 2022 and 2023 despite environmental, regional and national fish and wildlife agencies having found no evidence of environmental damage or adverse effects of net pen operations on wild fish. The National Oceanic and Atmospheric Administration (NOAA 2022) in a separate formal biological opinion held the same conclusion of no adverse effects of salmon net pens on endangered salmon and orca whales when net pens are operated and monitored under current regulations. Such unpredictability by government ministers tends to make fish farmers less likely to share details of operational problems including fish kills from HABs.

HAB prevention for fish mariculture is initially best accomplished by judicious site selection that focuses on using areas less likely to be affected by recurring HAB events. This means using locations with the best dispersion and assimilation of organic and inorganic waste produced without perturbation of benthic or water column habitats. Computer models are available to help site and size fish farms to minimize or eliminate adverse effects on sea bottom and water column habitats. However, in major fish farming countries, such options are limited due to existing fish farm development and hence other mitigation strategies are necessary. These approaches have been developed largely through trial and error by individual fish farming companies or by government assistance to small-scale fish farmers (e.g. Anderson et al. 2001).

Technical engineering studies of HAB mitigation methodology effectiveness, other than aeration or ox-

ygen supplementation, are limited compared to the major published literature dealing with mitigation or control of ectoparasites of fish in Norway, Canada, Australia and other regions. Technical analyses of flow fields and physical engineering of net cage operations have advanced greatly, but the quantification of HAB mitigation efficacy remains on a trial-and-error basis and includes technologies that have not been verified with appropriate data.

Here, we briefly discuss HAB mitigation strategies, highlighting categories that could be considered depending on the specific location and circumstance of each bloom event. Existing updated reviews of HAB prevention and mitigation for fish mariculture farms include Rensel and Whyte (2003) and Sellner and Rensel (2018). Some information herein has been condensed or updated.

9.1. Mariculture site selection and planning should occur prior to permitting actions to locate suitable areas for the concurrent benefit of the mariculture operation and the environment. This should include quantitative estimates of the risks of HAB recurrence. If pre-existing data do not exist, it is possible to design and size fish production to avoid contributing to coastal eutrophication using modelling tools to evaluate local to regional conditions. With proper siting and sizing of modern fish farms it is possible to have no adverse impact of net pen fish farming on benthic habitats, including but not limited to offshore habitats (Rensel et al. 2017). Particulate organic carbon in fish faeces and waste feed sink at known rates, and aquaculture models can calculate transport and assimilation into sediments and allow estimation of biological impacts.

It is possible to locate fish farms to not only limit benthic impact but to increase the biomass and diversity of benthic and epibenthic species in keeping with the Pearson-Rosenburg (1978) model of macrobenthos succession in relation to organic pollution. This is a generalized model because it is not predictive of changes at specific sites, a process that can best be achieved using computer models calibrated to local or regionally specific conditions. Water column eutrophication modelling is complex and involves far-field (3D) physical models coupled to phytoplankton population dynamics submodels that involve some knowledge and generalization of growth-limiting nutrient coefficients for a given species or groups of species.

Available computer models for site selection and planning include *www.aquamodel.net*, that accounts for carbon and nitrogen loading in nearfield 2D (single or clustered fish farms) and 3D (multiple farms over far field focusing on phytoplankton), developed for use and tested in numerous locations worldwide for Atlantic salmon and 12 other species of cultured fish. A 2D (single farm) model useful for Atlantic salmon was originally developed for Scottish lochs but is used elsewhere (*www.sams-enterprise.com/products/depomod)*. Many other fish mariculture models have been developed, but few were made available for use by non-computer specialists, fish farm managers or government agencies. These models are presently required to comply with government rules for new mariculture site description or existing site modification permitting in Chile, Canada, and Scotland. Summaries of other aquaculture models are available at NOAA's Coastal Aquaculture Planning Portal (*CAPP*) that presents a toolbox of coastal planning tools designed to assist managers, planners, and industry with sustainable aquaculture development.

Fish farms should be located in non-nutrient sensitive marine areas where nitrogen and less prevalent phosphorus waste will not contribute to existing or possible future coastal eutrophication. For example, in locations where sunlight penetration into the upper water column is minimal due to factors such as high latitudes or naturally occurring turbidity from rivers, the net effect is often that primary productivity is not nutrient limited. Examples of several fast-flushing seaward coastal channels that are not nutrient sensitive include seaward basins and channels of Washington State (Rensel Associates and PTI Environmental Services: US EPA 2001) and well-flushed channels or open-coastal bays of S.W. New Brunswick Canada (Harrison et al. 2005).

Alternatively, mariculture fish farms can mitigate their nitrogen production by coupling with seaweed or shellfish farms and other companion species first practiced as polyculture. In Japanese coastal bays, fish, shellfish, seaweed, and a variety of other seafoods have long been cultured together. But the scale of these marine farms is considerably smaller than large-scale corporate fish farms elsewhere and the extraction of waste to match input is not necessarily balanced. In Southern Chile, some mussel farms are interspersed among fish farms, but mussel farms can also produce inorganic dissolved nitrogen that can contribute to plankton bloom growth.

The use of integrated, multitrophic aquaculture (IMTA) is a possible solution for fish farm/eutrophication issues as it can capture dissolved and particulate waste products more effectively in a quantitatively designed approach. This methodology has been developing for many years in Atlantic Canada, Northeastern US, coastal Europe, Scotland, the Mediterranean, Australia and elsewhere (Soto 2010, Buck et al. 2018). While this can be desirable for diversification and environmental benefits, it has had limited adoption to date for existing large, near-shore fish mariculture production. If properly sited, benthic impacts are limited and ongoing clean-up afforded by IMTA is not necessary. IMTA research continues, and offshore applications in some regions are being planned or attempted.

Physical properties of fish farm sites to be avoided for eutrophication prevention include areas with extended periods of vertical stratification due to temperature or salinity, and slow water velocity and flushing rates that are often associated with enclosed embayments and fjords that may also have flow restrictions from sills near their entries. Biological considerations of eutrophication are reviewed by Borja et al. (2011) and nationwide surveys by NOAA and partners rank US coastal eutrophication by discrete regions (Bricker et al. 1999, 2007). Nevertheless, many existing small or large fish farms are currently located in nutrient-sensitive waters internationally.

In the northern region of the Chilean Inland Sea, both coasts of Chiloé Island and environs, net pen carrying capacity, i.e., the maximum volume of potential farmed fish production possible without causing algal blooms, is unknown but, in some subareas, existing operations may already be causing seasonal eutrophication and affecting HAB events (Anderson and Rensel 2017). Coupling of far-field circulation models to available biological models of aquaculture effects has been experimentally attempted in Chile, but its implementation would greatly advance understanding of the biological effects of the existing fish and shellfish culture industries. These methods allow classification of the nutrient status of inland sea subareas that can more effectively allocate production in specific subregions to avoid eutrophication. Quantitative analysis of production area carrying capacity is needed to inform discussions and lead to agreements among industry, government, and the public, while optimizing production efficiency. A detailed discussion is provided by Quiñones et al. (2019), but to date, no plan has been implemented to follow up.

Over the past two decades, some fish farming companies worldwide have elected to move mariculture operations further towards well-mixed, active channels and to the open ocean, both for the health of the fish and reduced risk to the environment. Such areas are often more capable of distributing solid waste (mainly organic carbon) and dissolved inorganic fractions (N and P) over broad distances and avoiding benthic eutrophication beneath the farms. Open-ocean fish farming is possible but involves greater capital expenditures and operating costs, though larger scales of production may offset these costs. Existing regulations and use conflicts for open-ocean fish mariculture have stalled demonstration farm permits, such as in Southern California.

Onshore (tank) fish farming, often using recirculating water with solid waste removal and conditioning, is practical in some areas for certain fish species. It is commonly used for extended juvenile rearing to allow the fish to become larger and to avoid known potential problems with excessively cold or warm water or time periods when risks of pathogens from wild fish are prevalent. Onshore fish farming for an entire fish production cycle is widely practised and has been successful in Norway, a country with

the largest open-water net pen production of salmon and rainbow trout. Yet in some other countries, onshore fish farming has had limited financial success for salmon and other species, and many failures have occurred due to catastrophic events and system failures, high construction and operation costs, and competition with lower cost production from net pens or capture fisheries.

In comparison, most large-scale net-pen fish farming efforts in coastal waters have been profitable since they became widely used in the 1980s. Siting practices, equipment and operational protocols have rapidly evolved. Governmental monitoring, third-party best practices rules and monitoring, major reductions of waste feed losses, fish disease avoided with use of vaccines, net cleaning, aeration or oxygenation support, and other improvements have improved efficiency and resulted in reduced waste nutrients per unit production. Large-scale net pen operations are now typically more prepared for avoiding HAB-caused fish mortalities, particularly in onshore or offshore farms that have access to deeper waters. Often major HAB events in any given location will not occur annually, and larger operations are insured against loss, but occasional destructive blooms and fish losses continue to occur, so there remains much room for improvement in prevention, control and mitigation of HABs.

9.2. Preemptive harvest is rarely a useful option for fish mariculture because the stocks of fish are too large to be quickly harvested, transported, and marketed prior to a bloom, even if several days or a week's notice of an impending bloom is provided. This applies to small and large-scale fish farms as post-mortem changes in fish tissues begin immediately after death and markets can only absorb a limited change in rate of acceptance, although if price is reduced, consumption could increase in some cases.

With perhaps a single exception (Florida *K. brevis*), none of the "ichthyotoxins" affecting farmed fish are of human health significance, meaning that HAB-killed fish are still fit for human consumption if they are fresh. Some public health authorities are often not well-informed about the safety of human consumption of farmed fish killed by HABs, and moreover the quality of recovered fish carcasses is typically poor, such that rendering and composting remains the most suitable option, especially for large volumes of killed fish. Mass fish mortalities may lead to tonnes of dead fish being dumped in landfills (Port Lincoln, Australia 1996) or dumped offshore (Chile 2016). In Washington State and British Columbia, fish farm mortalities including routine minor losses have been sold for fertilizer production, but some may have been buried in landfills.

9.3. Fish feeding cessation during HAB events is recommended in most cases and is to be maintained if no other effective mitigation method is available. Fish metabolic oxygen demand increases significantly during and after feeding during digestion, and respiration may be impaired if gills become damaged or clogged by algal cells and gill mucus following exposure to HAB cells. Over prolonged periods of several weeks this practice causes increased physiological stress due to reduced liver glycogen, catabolism of tissues with associated weight loss, resorption or impairment of developing gametes, and an increased susceptibility to bacterial or viral diseases.

9.4. Aeration or oxygenation of water is widely practised in fish farms, usually to offset high loading densities of fish or low ambient levels of dissolved oxygen that can lead to stress, disease, or reduced growth. In some cases, aeration systems are configured to start automatically when monitoring equipment detects within-cage oxygen concentrations that have dropped below a given threshold that varies among species. Aeration may also be useful in some cases for mitigating the physiological effects of some HABs that cause environmental hypoxia or anoxia (i.e., large-biomass blooms of noxious or normally benign microalgae that may be respiring at night or have become senescent and decaying). Oxygenation uses similar dispersion equipment but uses pure oxygen from storage containers or on-site production and is much more effective in keeping fish alive than aeration, but super-

saturation must be avoided to prevent other problems. Neither aeration nor oxygenation alone will typically prevent lethal effects of fish exposure to several species of HABs. These methods are therefore usually considered important supplements to other methods that aim to reduce direct exposure to fish-killing HABs.

Different variations of aeration include microbubble aeration, venturi nozzle systems, hypolimnetic destratification, surface agitators, fountains, splash aerators, vertical pumps, and paddlewheels to enhance the transfer rate of oxygen from the atmosphere to the water. Venturi nozzle aeration has been used successfully to remove dense surface slicks of *Noctiluca* in Tasmanian fish farms (Hallegraeff et al. 2019). Aeration alone is often insufficient to protect cultured fish from HAB events in cage culture for several reasons, including that the increase in oxygen concentration is often minimal compared to the needs of the fish. Methods that prevent contact of HABs with fish are more effective mitigation strategies, as even small-scale exposures can cause growth impairment, secondary infections by bacteria or viruses, or in the case of brood stock, production of defective gametes.

9.5. Airlift Upwelling is one of the most widely used methods in fish mariculture to mitigate HAB effects. It can be an efficient method to pump deeper water to displace HAB cells in surface waters within fish net cages. This methodology is sometimes used alone or can be combined with other approaches such as oxygenation (discussed previously) or perimeter skirts and bubble curtains (discussed below).

A prerequisite for airlift aeration to function properly is sufficient depth to provide deep water that has reduced HAB abundance and that it is not low in oxygen (< 5 mg/L for salmonids). In other words, if a bloom is surface-oriented to approximately 15- or 20-meter depth like the depth of many commercial net pens, then the deep water is used to displace the surface water and its algal load. In some cases, HABs are not surface-oriented and repeated monitoring of algal biomass at depth must be practised to avoid pumping HAB cell-laden deep water into the cages.

Initially some fish farmers used large diameter, corrugated drain tubing to direct the upwelling but this was cumbersome and became a surface for biofouling. In some locations, the tubing was discarded in favour of simply routing the compressed air hose below the middle of the cages allowing the water to flow upward to create a supply of deep water into the cages. In British Columbia and Washington State, many fish farm sites are in areas with strong current velocities, and the net gauges are large enough to receive the upwelled water without excessive lateral dispersion. In one case, fish farm managers visually judged the slight variation in coloration of upwelled water to achieve optimum depth of airlift initiation to successfully mitigate a *Heterosigma* bloom. The visual method was validated to be accurate using an *in situ* fluorometer (Rensel 2007), but fish farms can be large, and plankton are by nature patchy in distribution. Fish farm operators must be vigilant during HAB events, and visual methods will not be sufficient at night when a submersible fluorometer sensor would be useful (if the bloom is not a mixture of benign and HAB species) or if repeated wet-mount cell counts are conducted. Newer technologies like the Imaging Flow Cytobot (IFCB; Olson and Sosik 2007), a submersible, automated microscope, can provide *in situ* phytoplankton community identification and analysis. If an instrument of this type is lowered through the water column, vertical profiles of HAB species can be obtained in near real time.

Airlift upwelling often leads to added aeration, but subsurface layers may have less dissolved oxygen than surface layers. If this is severe, it is a factor that should have been determined before a mariculture site was developed. If not, it may be managed through additional aeration or oxygen supplementation. A companion technique referred to as cage perimeter skirts may be deployed around the cages to prevent surface-oriented HABs from entering fish cages, particularly at sites with relatively slow current velocities. With high fish biomass, oxygenation may likely be required concurrently.

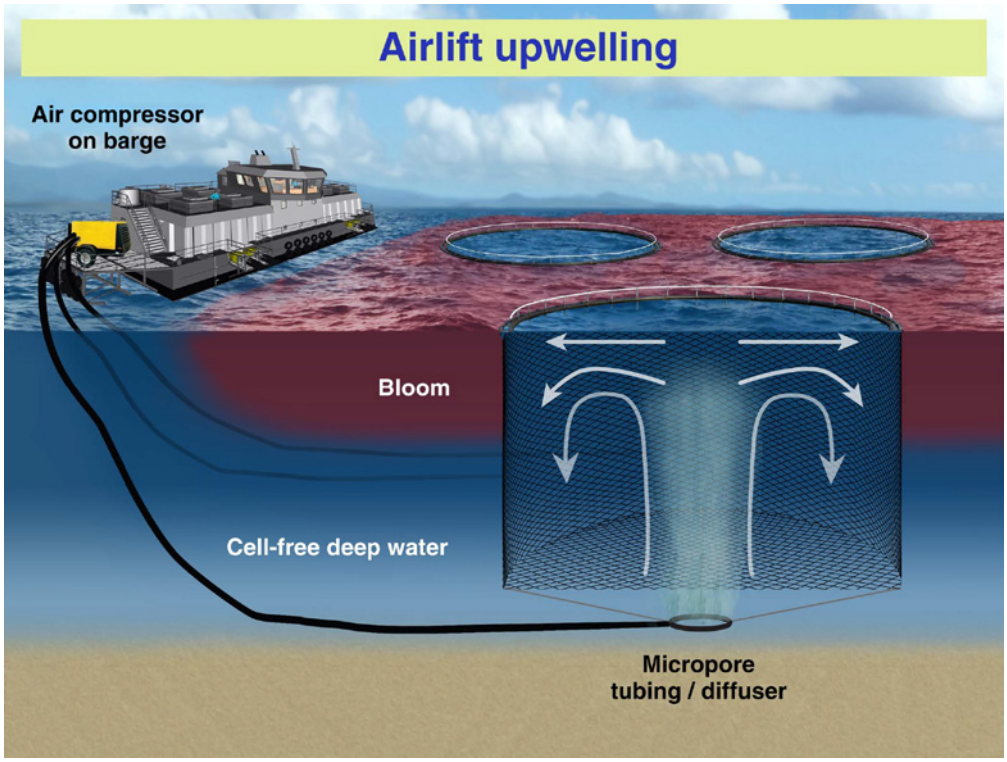

Figure 9.1. Diagram of airlift-upwelling system used to displace surface-oriented algal blooms from within fish cages using cell-free deep water; shown here with an optional vertical perimeter skirt around the cage and a compressor on a feed handling barge (from Sellner and Rensel 2018).

In 2018, major salmon losses were reported at two fish farms in Sechelt Inlet, British Columbia from a *Heterosigma* bloom. Few details were available to the public except an aquaculture trade magazine reporting that the bloom occurred well below normal surface depths in this slowly flushed inlet. In this instance, the upwelling system did not work properly (although it had in the past), resulting in the loss of 1,000 metric tons of salmon (IntraFish Media 7June 2018) or about half of the stock being reared. *Heterosigma* are more likely to be toxic when the cells become nutrient stressed and begin to sink (Powers et al. 2012), which may explain what happened in Sechelt Inlet.

It is unusual for *Heterosigma* to occur in major concentrations at deep, subsurface depths but not uncommon to be in layers near a temperature and salinity discontinuity, including thin layers, but details regarding this fish kill, like many others, were not provided by the grower. Relatively rare blooms of *Margalefidinium polykrikoides* in British Columbia also frustrated attempts to use airlift upwelling as the cells of this species were found much deeper than is common with *Heterosigma* and may have contributed toxin at a greater rate than normal due to cell disruption by the turbulence involved with upwelling (Whyte et al. 2001). Park et al. (2013) report the use of airlift upwelling in South Korea and it has also been applied in other countries.

During the largest fish kill in aquaculture ever recorded, a massive bloom of *P. verruculosa* in Southern Chile in 2016, air-lift upwelling was thought to be ineffective (Mardones et al. 2021). Method efficiency may have been limited because the depth distribution of the HAB at some farms may not have been closely tracked to adjust the depth of air-lift pumping. The cells may have been deeper in the water column than expected, and thus been unintentionally pumped into the cages. Chlorophyll vertical profiles showed large concentrations of the alga in thin layers associated with a strong thermocline and halocline at about 10m depth. Variable intake depth and increased pumping power could likely have been more effective to displace a bloom located midway between the surface and bottom of net pens.

Figure 9.2. Aerial photograph of net pens with airlift upwelling during a *Heterosigma* bloom in British Columbia showing dramatic colour difference in inside pens versus outside pens. Photo courtesy of Cermaq Canada Ltd. as published in Sellner and Rensel (2018).

If HAB density is sufficiently high, some fish farmers have visually altered the depth of pumping until the water appeared clearest. A Turner *in vivo* chlorophyll sensor thus was used successfully by the second author during a fish-killing *Heterosigma* bloom in North Puget Sound. Cells were being transported in the lowered salinity plume of the Fraser River and no diel depth variation was observed but the fish farm manager was able to visually adjust the depth of the air-lift pump and his choices were validated as optimum with the fluorometer (Rensel 2007).

9.6. Bubble Curtains are created by using a ring of perforated tubing through which a compressor pumps air, with a flexible lead weight to counteract buoyancy. These are submerged around the perimeter at depth by the net pens. The mechanism of possible protection from HABs is thought to be a physical barrier around the net pen perimeter to prevent entry into the pen system. This might be effective for quiescent areas, but in strong current areas needed for larger fish net pen systems, it is unlikely to divert water and cell flow. Depending on the volumes and type of bubbles, it may create turbulence sufficient to damage some of the more delicate HAB cells, and this could release toxic compounds. For *Heterosigma* this seems unlikely, however, as lysing of cells has not been found to increase fish toxicity in fish bioassays. For harmful diatoms, Rensel (1993) found that shorter chains of *Chaetoceros* (*Phaeoceros*) cells produced by stirred cultures were less harmful to juvenile Atlantic salmon compared to those of stationary cultures while considering that both treatments had the same total number of cells in live fish bioassays.

Similar to airlift upwelling, it is important to monitor HAB depth distribution or the use of bubble curtains could also transport deeper-occurring HABs upwards to adversely affect the cultured fish. Bubble curtains have been used in British Columbia with *Heterosigma* in some locations, including slowly flushed and vertically stratified Sechelt Inlet, discussed above, and in the Inland Sea of Southern Chile that has similar locations, as well as open, well-flushed waters, and in coastal areas of South Korea for *M. polykrikoides* (Kim 2012). The efficacy of bubble curtains has been questioned by Mardones et al. (2021) in Chile where it was used but judged ineffective, possibly because the depth distribution of HAB cells was not routinely monitored by the fish farms to adjust depth of the equipment.

While published research regarding bubble curtains for mitigating HABs is rare, a recent study of bubble curtains to reduce jellyfish entry into fish farms (Habrlin et al. 2021) concluded, as we have,

that slowly flushing areas of weak currents are probably a more suitable venue for this methodology. But otherwise, moderate-to-strong currents (e.g., mean horizontal velocity of 10 to 20 cm sec^{-1}) that exist at many large fish farms sites may prevent bubble curtain effectiveness. It is also of interest that these same authors determined that a bubble curtain barrier set at an acute angle to rectangular cages is more effective than a perpendicular angle.

9.7. Perimeter Skirts, also known as perimeter tarpaulins, have long been used for different purposes, most recently in locations where sea lice, which are surface-oriented, can infect salmon or other cultured fish. In areas without sea lice, perimeter skirts made out of fine mesh material have been used to reduce the concentration of larval crab that have sharp spines that can lacerate the salmon gills. If impervious perimeter skirts are used, the concentration of dissolved oxygen must be constantly measured and possibly supplemented depending on fish density. Impervious skirts were mentioned above as commonly used in combination with aeration or upwelling aeration.

9.8. Closed-containment floating pens are like net pens but have solid, impervious vertical walls and a closed bottom and rely entirely on pumped water to keep fish alive and remove solid wastes. Some have suggested a floating, closed-containment net pen could be a solution to HAB problems in marine fish aquaculture. There have been several trials of such systems but none have been commercialized as they are energy intensive compared to net pens, more difficult to anchor successfully due to tidal current stress and collected fish wastes containing salt residue - a further expensive complication if the wastes are to be reclaimed and used onshore as fertilizer. Opponents of closed containment fish aquaculture note that HABs do not occur annually at properly cited fish farms and may only be a once in five-to-ten-year event. Nevertheless, closed-containment pens have been tried and failed repeatedly as an efficient or cost-effective means to rear fish. Two examples are provided.

In Tasmania, Australia, small scale closed-containment pens were tested in a terminal inlet bay of the Huon Estuary during austral summer of the year 2000. An automated chlorophyll monitoring and vertical profiling system was used to relocate the pump intake to different depths. The waters were replete with several different potential fish-killing HAB species at all depths, however, and no single depth was HAB-free at the shallow (15-20m) location. Oxygenation was used but not very effective in preventing loss of the Atlantic salmon.

In 1974, the Weyerhaeuser Co. and Washington State Sea Grant collaborated on placement and operation of a fish farm near a log rafting area in shallow and slowly flushed Southern Puget Sound. Net pens and closed containment, floating raceways were used to rear coho salmon, and spot prawns *Pandalus platyceros* were reared under the net pens (Rensel and Prentice 1979). Recurring blooms of the dinoflagellate *Tripos fusus* killed large numbers of the fish and prawns in surface waters. The spot prawns had blackened gills with suctorian protozoan (secondary) infections but were unaffected at a central Puget Sound net pen location that was deeper, cooler and had strong tidal flows. Again, shallow, restricted bays and inlets are not suitable for any type of commercial fish mariculture including closed containment cages.

9.9. Moving net pens from an area affected by a HAB event to a known refuge area may be an effective mitigation measure and is a preferred method in some regions. It does, however, present a considerable risk and expense for larger systems, although a fish-farming insurance company may offset part of the expense. Risks include grounding or tearing of net pen mesh or structural collapse of cage floatation that may not tolerate the added stresses of towing and tidal currents concurrently.

Towing away from HAB events has been used in several locations including for mitigation of the harmful dinoflagellate *Karenia mikimotoi* in Norway, *Chattonella antiqua* in the Seto Inland Sea of Japan and in Hong Kong Harbor (reviewed by Anderson et al. 2001; Sellner and Rensel 2018). More recently

it was used for some facilities in Southern Chile in the 2016 massive bloom and judged to be the most effective mitigation method attempted during that event (Mardones et al. 2021).

In countries with large fish-farming companies and operations, moving large farms is often not feasible due to net pen facility design, costs and risks of escaped fish or damage to cages. However, if commercial shipping and other large vessel use nearby a pen moving operation is minimal and the weather and tides are mild, moving large net pens may be worth attempting. Similarly, areas with large number of small, family-operated farms may encounter significant adverse effects on other aquatic uses and industries, such as interference with marine shipping that was encountered during a major fish kill in Hong Kong (Anderson et al. 2001).

Selection of a potential target refuge area should occur far in advance of any attempted towing operation. Towing net pens away from a HAB has been used a few times in preventing cultured fish losses due to *H. akashiwo* in Puget Sound, Washington State. When last tried, strong tides on the pathway to a refuge area resulted in cage damage and loss of some Atlantic salmon. The refuge area, known as Colvos Passage near Vashon Island was also known to have strong vertical mixing that would inhibit or kill *Heterosigma* cells. Escaped Atlantic salmon do not survive to reproduce along the North American west coast or compete with Pacific salmon but at the time of this event, that fact was not well-established.

9.10. Delayed or changed seawater entry timing of juvenile fish to be planted in net pens has not previously been considered as a HAB mitigation method. After years of fish farming operation, known risk periods can often be identified and juveniles of some fish species (e.g. Atlantic salmon) can be stocked in seawater during different seasons to avoid times of increased risks such as HAB events or excessively cold or warm conditions. In some locations, postponement of seawater entry can help avoid exposure to marine ectoparasites, such as crustacean-copepod sea lice. Shorter time periods in seawater also reduce the risks of marine HABs affecting fish with altered seawater entry timing.

9.11. Flocculation. Flocculation is the process of removing microscopic algae using clay to create algal sedimentation. Through repeated collisions and adhesion, large, rapidly sinking aggregates (or flocs) of algae and clay are formed and settle to the ocean floor (Figure 9.3). Many different types of clay have been tested, with some having a much higher flocculation efficiency for algal cells (Sengco et al. 2005). For example, Rensel et al. (2004) found high removal efficiency (84%) of *H. akashiwo* in mesocosms treated with only 0.12 g/L phosphatic clay. "Ball" clay (20-80% kaolinite, 10-25% mica, and 6-65% quartz) could remove >90% of *Pyrodinium bahamense* and *Gymnodinium catenatum* in Philippines coastal waters (Padilla et al. 2007; Rivera 2015). Clays rich in titanium dioxide appear to be 20% more effective, generating reactive oxygen at light levels >2300 µmol m^2 s^1 versus dark rates (Kim et al. 2001). Some clays can also absorb and sequester HAB toxins (Pierce et al. 2004).

The use of clay to flocculate and settle HAB organisms to bottom sediments has been successfully used at large scales for over 20 years in Asia. In South Korea, clay has been dispersed since 1996 to control blooms of the fish-killing species *Margalefidinium polykrikoides*. In some years, an astounding 100,000- 200,000 tons of clay have been dispersed (Park et al. 2013). In 1996, the first year of the clay applications, fisheries losses dropped from to USD 1M from USD100M the year before. In subsequent years, losses have been reduced similarly. In terms of environmental impacts in South Korea, paraphrasing Park et al. 2013, ecosystem impacts due to clay dispersion, particularly the benthos, have been assessed since 1998. No significant differences in biomass or species composition of the benthos such as Annelida, Mollusca, Decapoda, or Arthropoda have been observed between the areas of clay dispersal and control areas." (NFRDA 2008, cited in Park et al. 2013). Furthermore, numerous HAB mitigation methods have been examined in Korea, including clay, marine bacteria, microscreen

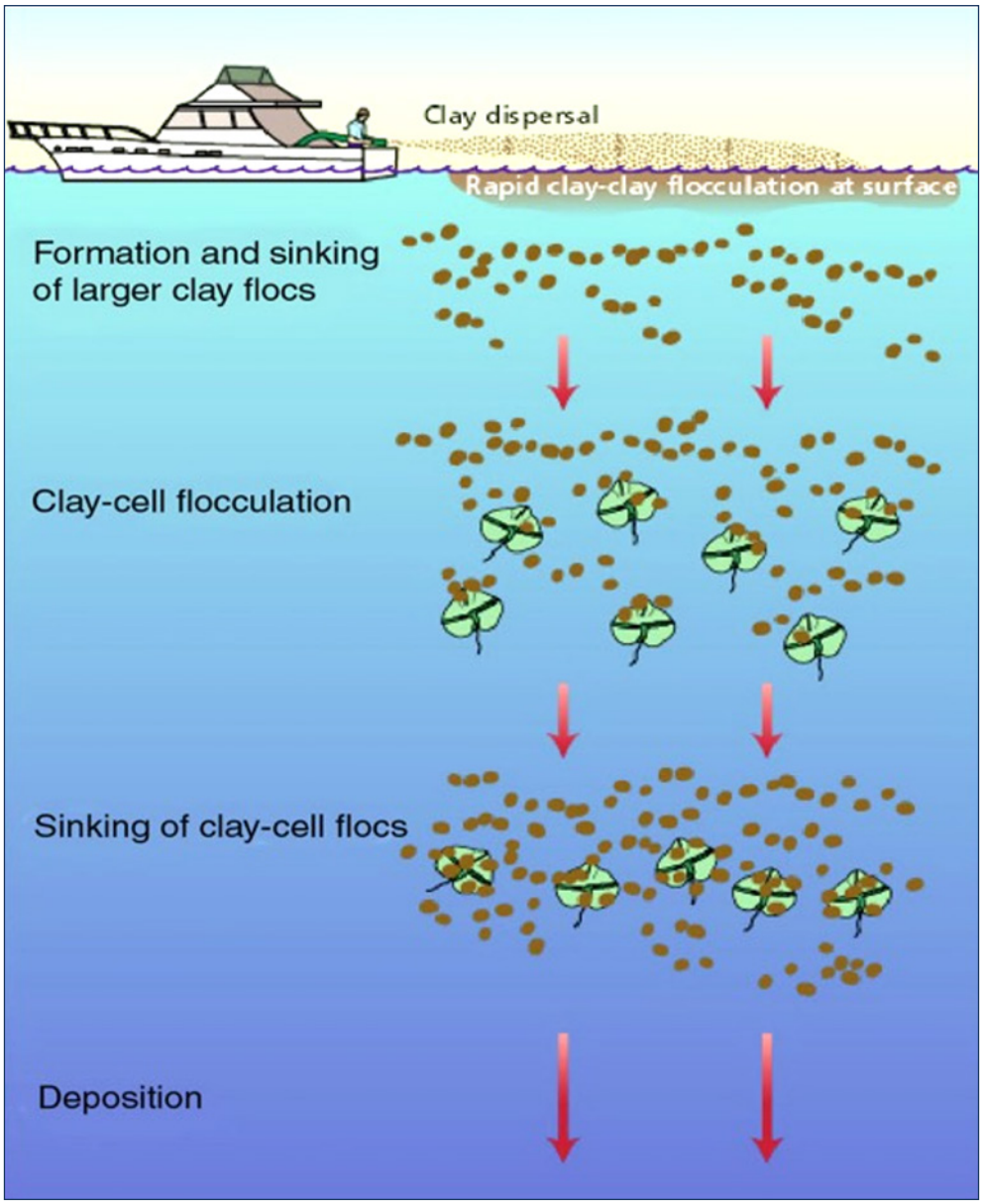

Figure 9.3. Clay dispersal and flocculation, leading to removal of HAB cells (adapted from Sengco et al. 2005).

filtration and ozone, ultraviolet radiation, parasitic dinoflagellates, and microzooplankton predators of bloom species. Nevertheless, no other control methods have been used extensively in the sea except clay (Park et al. 2013). Clay dispersal is now a routine HAB management strategy to protect South Korean fish farms.

The Chinese have also used clay for bloom control in coastal waters for over 10 years (Yu et al. 2017), though the focus has not been on fish farms, but rather on nuclear power plant intakes, tourist beaches, or other sites for recreational activities. One treatment prior to the sailing events in the 1998 Olympics covered 100 km^2, and since then, more than 20 large-scale clay treatments have occurred.

A major reason that clay dispersal is considered by many to be the most attractive and scalable of all marine HAB control strategies stems from breakthroughs by Chinese researchers that led to a modification of clay minerals that greatly reduces the amount of clay applied. The key is a highly purified kaolinite clay that has been modified to include an inorganic polymer called polyaluminum chloride (PAC). PAC is used routinely in water purification and lake restoration, meets American Water Works Association standards (ANSI/AWWA B408-03), and is certified in the US by the National Sanitation Foundation (NSF) for use in treating drinking water at a maximum dose of 250 mg/L. For HAB control,

PAC is typically present at < 5 mg/L. Treatments of unmodified Korean clay (yellow loess) require clay loadings of 400 g/m^2 (Park et al. 2013), but for the Chinese modified clay (hereafter MC), loadings are 50 - 100 times lower, or 4 -10 g/m^2 (4-10 tons/km^2) (Yu et al. 2017). This is roughly equivalent to two packets of sugar spread over one square meter. Korea has also greatly reduced clay loadings, in their case using electrolysis of ambient seawater to produce sodium hypochlorite and other residuals, and subsequent mixing with local clays (Park et al. 2013). Consistent with Korean findings, Chinese workers report no negative environmental impacts from clay flocculation (Lu et al. 2015; Gao et al. 2007; Wang et al. 2014; Yu et al. 2017). A recent review by Song et al. (2021) stated that "Concerning the impacts of MC on aquatic organisms, the results showed that MC has no adverse effects on fish, shrimp, or most shellfish……"). Further testing of clay should cover different aquaculture operations (large vs small scale, deep vs shallow waters) subject to different hydrological conditions.

While clay flocculation initially focused on removing algal cell biomass, Seger et al. (2017, 2021) instead targeted applications of clay for ichthyotoxin adsorption. Bentonite clay could eliminate *Prymnesium, Kareni*a and *Karlodinium* ichthyotoxicity at bentonite clay loadings of 0.05-0.25 g/L, significantly lower than those considered to be harmful to benthic marine invertebrates (1-10 g/L; Shumway et al. 2003).While extracellular *Chattonella, Heterosigma* and *Alexandrium* ichthyotoxins could only partially be removed, early application of these clays during the process of cell lysis eliminated ichthyotoxicity.

In summary, some countries such as South Korea and China are making significant advances in HAB mitigation or control, but most western countries are moving slowly due to environmental concerns and cautions. Some approaches are effective on a small scale (e.g., within a fish net pen or a residential canal) whereas others are scalable to larger areas. Fish farms have a range of options to consider for HAB control. Some require localized approaches in the immediate area of net pens (e.g., airlift upwelling, perimeter skirts), whereas others can be applied further away, in hopes of stopping a HAB before it reaches the pens. The latter approaches are necessarily larger in scale, greatly limiting the treatment options due to cost and logistics. If this were to be attempted (such as at the mouth or entrance to a fjord or embayment), clay dispersal is presently the most cost effective and environmentally benign.

HABs are disasters for aquaculture, and should be treated as such. There will be some environmental impacts from bloom control strategies, but those impacts need to be compared to the impacts of the untreated HAB, which can be catastrophic. The public, politicians and aquaculture industry in most countries desire and expect some level of HAB control, so research and field trials with open-water treatments need to be sustained and expanded.

Priority topics for research and implementation

- Explore multiple and combined bloom control strategies - no single method will be suitable for all HABs or locations.
- Sponsor and promote field and large-scale tank research to quantitatively assess effectiveness of HAB mitigation procedures for fish farming.
- Promote sharing of fish farming mitigation results among industry and researchers and governments, especially those that make special efforts to protect and embellish fish farming (e.g., Hong Kong, Singapore).
- Further explore methods for both cell and dissolved toxin removal; consider clays combined with adsorbent or oxidizing materials that can sequester or destroy specific ichthyotoxins.
- Move rapidly from laboratory-based experiments to pilot- and larger-scale studies with sound experimental design to quantify effectiveness and environmental impacts.
- Work with regulatory agencies to streamline permitting processes for experimental and operational bloom control studies. Most environmental agencies are not accustomed to receiving applications for the use of clay, chemicals, or organisms for HAB control, and in many cases, appropriate regulations do not exist.
- Work with social scientists to help build community and political support and involvement; engage fishers and other aquatic farmers in mitigation studies.

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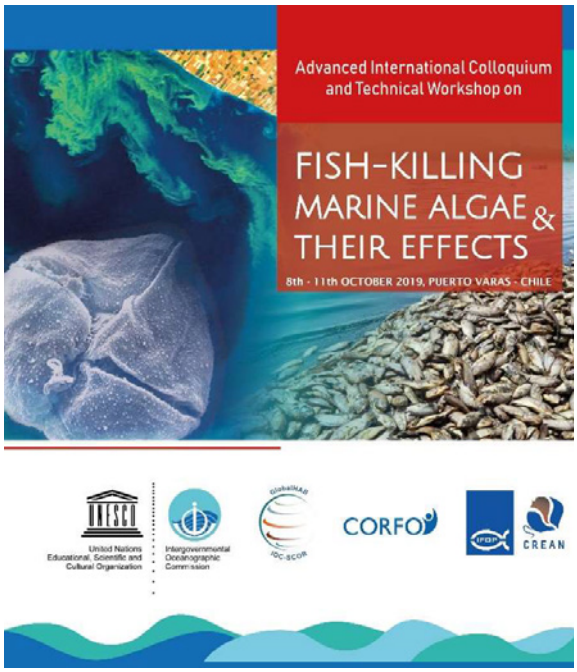
APPENDIX 2: MEETING PROGRAM



Venue: Puerto Varas, Chile
Dates: 8th – 11th October, 2019

Fish-killing algal blooms are of increasing concern to socio-economic interests linked to the sustainability and security of seafood and living resources. Development of fisheries and aquaculture as part of integrated coastal resource management are particularly susceptible to the threat of ichthyotoxic events and their consequences. Whereas these events are categorized as "fish-killing", there are associated impacts on other components of coastal marine ecosystems, including wild fish populations, benthic macrofauna and macrophytes. Outside the aquaculture and fisheries industry sector, there has been inadequate consideration of fish-killing algae and the topic has not been systematically addressed within the scientific community on a global basis. Known ichthyotoxic marine algae are usually identifiable as causative organisms, but there remain taxonomic and biogeographical uncertainties in the distribution of the species. The role of climate change leading to regime shifts and hence possible increased frequency, magnitude and biogeographical distribution of fish-killing algal blooms poses a challenge to understanding the future ocean. Knowledge of the environmental factors driving bloom dynamics are not fully understood, and this has hampered the development of predictive models for forecasting and risk assessment of fish-killing events. Even the proposed mechanisms whereby exposure to such blooms causes fish morbidity and mortalities are highly controversial and lack scientific consensus. Furthermore, there is only limited application and lack of standardization of current fish- or cell-based bioassay methods for assessing ichthyotoxicity. The Advanced International Colloquium and Technical Workshop, under the auspices of the IOC-IPHAB and as part the GlobalHAB science agenda, with the support of the government of Chile through CORFO and the collaboration of CREAN-IFOP, will comprehensively address these gaps in knowledge and will yield a synthesis of current state-of-knowledge linked to strategies for technological and scientific approaches to mitigating the impacts. As a way to strengthen the relationship with the local community, part of the considered activities in this Colloquium, a set of talks oriented to decision makers, professionals, academics and the general public, has been included.

GENERAL PROGRAMME & SESSION THEMES

Taxonomy, biogeography, ecology, oceanography and dynamics of fish-killing algal blooms: relationships to fish mortality events

Topic 1: Climate change and fish-killing algae
Topic Coordinator: Gustaaf Hallegraeff, Australia
Key Issues: An exploration of the extent to which fish-killing algal events can be linked to climate changes in the recent past and scenarios for the future changing ocean.

Topic 2: Taxonomy and molecular characterization of fish-killing algae
Topic Coordinator: Mitsunori Iwataki, Japan
Key Issues: New taxonomic descriptions and phylogenetic reconstruction of fishkilling algal taxa based upon morphological and molecular criteria.

Fish-killing toxins, aetiology and specific mechanisms of fish morbidity and mortality

Topic 3: Current knowledge of ichthyotoxins produced by fish-killing microalgae
Topic Coordinator: Thomas Ostenfeld Larsen, Denmark
Key Issues: Developments in analytical and natural products chemistry leading to discovery and structural elucidation of fish-killing toxins: links to chemodiversity.

Topic 4: Mechanisms of algal-induced fish-killing syndromes
Topic Coordinator: Per Juel Hansen, Denmark
Key Issues: Critical analysis of putative mechanisms for cell damage in fish-killing algal events – effects of ichthyotoxins, membrane disruptors, haeomolysins, etc.: links to chemical ecological function in the producing algae.

Topic 5: Development and validation of current fish- or cell-based bioassay methods for assessing ichthyotoxicity
Topic Coordinators: Hélène Hegaret, France (and Jorge Mardones, Chile)
Key Issues: Comparison of functional bioassays for ichthyotoxic assessment of wholecell versus cell-fraction exposure: strengths and weaknesses of alternative techniques and potential for optimization and standardization of protocols.

Management and mitigation of fish-killing algal events

Topic 6: Impact of fish-killing algal events on other components of coastal marine ecosystems
Topic Coordinator: Lincoln Mackenzie
Key Issues: Consideration of the broader ecological impacts of fish-killing algal blooms, including effects on wild fish populations, benthic assemblages, and other components of the pelagic zone: multi-stressors induced by nutrient deprivation or local organic enrichment via degradation, deoxygenation, released toxin components, or disruption of food web interactions.

Topic 7: Assessment of mitigation strategies and their effectiveness
Topic Coordinator: Don Anderson, USA
Key Issues: Current application and future opportunities for deployment of technological solutions for monitoring fish-killing algal blooms and mitigating their effects on fish in aquaculture operations and wild populations, e.g. early warning systems, physical displacement of cages, application of clay or other flocculants, physical enclosure with pumping (oxygenation), etc.

CLOSED SESSIONS

Tuesday, 8th October (Day 1)

08:30 – 08:45 Welcome words Leonardo Guzmán
08:45 – 09:00 Opening Allan Cembella
09:00 – 09:35 Topic 1 (35 minutes for talk)
09:35 – 09:55 Key questions Topic 1 (20 minutes for questions and discussion)
09:55 –10:30 Topic 2 (35 minutes for talk)
10:30 – 10:50 Key questions Topic 2 (20 minutes for questions and discussion)
10:50 – 11:10 Health break
11:10 – 11:45 Topic 3 (35 minutes for talk)
11:45 – 12:05 Key questions Topic 3 (20 minutes for questions and discussion)
12:05 - 12:40 Topic 4 (35 minutes for talk)
12:40 – 13:00 Key questions Topic 4 (20 minutes for questions and discussion)
13:00 – 14:30 Lunch
14:30 – 15:25 Discussion Topic 1 (55 minutes)
15:25 – 16:20 Discussion Topic 2 (55 minutes)
16:20 – 16:40 Health break
16:40 – 17:35 Discussion Topic 3 (55 minutes)
17:35 – 18:30 Discussion Topic 4 (55 minutes)

Wednesday, 9th October (Day 2)

09:00 – 09:35 Topic 5 (35 minutes for talk)
09:35 – 09:55 Key questions Topic 5 (20 minutes for questions and discussion)
09:55 –10:30 Topic 6 (35 minutes for talk)
10:30 – 10:50 Key questions Topic 6 (20 minutes for questions and discussion)
10:50 – 11:10 Health break
11:10 – 11:45 Topic 7 (35 minutes for talk)
11:45 – 12:05 Key questions Topic 7 (20 minutes for questions and discussion)
12:05 - 13:00 Discussion Topic 5 (55 minutes)
13:00 – 14:30 Lunch
14:30 – 15:25 Discussion Topic 6 (55 minutes)
15:25 – 16:20 Discussion Topic 7 (55 minutes)
16:20 – 16:40 Health break
16:40 – 17:35 Discussion Topic 7 (55 minutes)
17:35 – 18:30 Introduction. Overview topics 1 to 7. Synthesis/future perspectives discussion. (55 minutes)
Evening 20.00: Workshop dinner

Thursday, 10th October (Day 3)

09:00 – 11:20 Meeting to organize report, special issue, book or compilation. Outlines for future fish-killing workshops and/or events (Part 1)
11:20 – 11:50 Health break
11:50 – 13:00 Meeting to organize report, special issue, book or compilation. Outlines for future fish-killing workshops and/or events (Part 2)
13:00 – 14:00 Lunch

OPEN CONFERENCE

Status of fish kills and its impacts in a changing environment

(this activity will be open to professionals of the Fisheries and Aquaculture Undersecretary, Fisheries and Aquaculture National Service, researchers and students from Universities, and sectorial local authorities, including professionals from the salmoniculture sector, and interested people)

Simultaneous translation plus real time streaming will be available

14:00 – 14:30	Registration
14:30 – 15:45	**Per Juel Hansen & Thomas Ostenfeld Larsen.** Overview: fish killing mechanisms, ichthyotoxins, detection and identification methods and its standardization. Presentation + Questions (35 minutes for talk and 10 minutes for questions)
15:45 – 16:30	**Gustaaf Hallegraeff**. Climate change and fish killing algae. Presentation + Questions (35 minutes for talk and 10 minutes for questions).
16:30 – 16:50	Health break
16:50 – 17:35	**Don Anderson.** Current mitigation strategies and their effectiveness. Presentation + Questions (35 minutes for talk and 10 minutes for questions).
17:35 – 17:55	**Paulina Artacho**. HABs monitoring management in the Chilean salmon industry: advances and challenges. Presentation + Questions (15 minutes for talk and 5 minutes for questions)
17:55 – 18:20	**Alejandro Clément**. Distribution of HABf INDEX and Analysis of the Economic Impacts in Salmon Farming. Presentation + Questions (20 minutes for talk and 5 minutes for questions)

Friday, 11th October

08:00 - 13:00	Salmon farm visit
13:00 - 14:30	Lunch
	Departure