RGC Ref. No.:
UGC/FDS16/M01/18
(please insert ref. above)

RESEARCH GRANTS COUNCIL
COMPETITIVE RESEARCH FUNDING SCHEMES FOR THE LOCAL SELF-FINANCING DEGREE SECTOR

FACULTY DEVELOPMENT SCHEME (FDS)

Completion Report
(for completed projects only)

Submission Deadlines:
1. *Auditor's report with unspent balance, if any: within **six** months of the approved project completion date.*
2. *Completion report: within **12** months of the approved project completion date.*

Part A: The Project and Investigator(s)

1. Project Title

Molecular Responses of Fish Gills Experimentally Exposed to Ichthyotoxic Dinoflagellate *Karenia mikimotoi*

2. Investigator(s) and Academic Department(s) / Unit(s) Involved

Research Team	Name / Post	Unit / Department / Institution
Principal Investigator	LEE Wang Fat / Professor	School of Science and Technology, Hong Kong Metropolitan University
Co-Investigator(s)	XU Jing Liang / Associate Professor	School of Science and Technology, Hong Kong Metropolitan University
Others	N.A	N.A

3. Project Duration

	Original	Revised	Date of RGC / Institution Approval *(must be quoted)*
Project Start Date	1 January 2019	N.A	N.A
Project Completion Date	31 December 2020	30 June 2021	11 March 2020
Duration *(in month)*	24	30	11 March 2020
Deadline for Submission of Completion Report	31 December 2021	30 June 2022	11 March 2020

Part B: The Final Report

5. Project Objectives

5.1 Objectives as per original application

1. ***analyse*** the phylogenetic relationship between the locally isolated *Karenia mikimotoi* strain and other strains originating from different geographical locations
2. ***optimise*** the growing conditions of the cell culture of *K. mikimotoi*
3. ***evaluat****e* the effects of growth phases on the ichthyotoxicity of *K. mikimotoi*
4. ***determine*** the median lethal time (LT50) of the ichthyotoxicity of *K. mikimotoi* on the viability of both *in vitro* fish gill cells and *in vivo* medaka fish
5. ***compare*** and ***analyse*** the protein expression profiles of fish gill cells (*in vitro* and *in vivo*) upon exposure to *K. mikimotoi* in terms of different time of exposure and algal cell concentrations as guided by the experiments performed in objectives #3 and 4
6. ***identify*** differentially expressed proteins in different comparative conditions performed in objective #5, and ***validate*** the differential protein expressions using immunoblotting and/or real-real-time PCR techniques
7. ***elucidate*** the possible molecular pathways by which fish gill cells are exposed to *K. mikimotoi*

5.2 Revised objectives

Date of approval from the RGC: N.A

Reasons for the change: N.A

5.3 Realisation of the objectives
(Maximum 1 page; please state how and to what extent the project objectives have been achieved; give reasons for under-achievements and outline attempts to overcome problems, if any)

All objectives were completed smoothly without major problems. The realization of the objectives is summarized below.

Objective 1: We first conducted literature review and optimized our experimental conditions by referring to a previous study "Al-Kandari, M. A.; Highfield, A. C.; Hall, M. J.; Hayes, P.; Schroeder, D. C., Molecular tools separate harmful algal bloom species, *Karenia mikimotoi*, from different geographical regions into distinct sub-groups. *Harmful*

Algae 2011, 10, (6), 636-643. Three DNA regions of the locally isolated *Karenia mikimotoi* strain were isolated and amplified. Phylogenetic analysis was performed by comparing the three DNA regions of our local stain to other *Karenia mikimotoi* strains that isolated from different geographic locations. Our result indicated that KMHK clusters within the Japanese and Chinese clade (with >98% identities), and the genetic distance of KMHK was closely affiliated to the KM IV strains isolated from South China Sea

Objective 2: Effect of different growing conditions (cultivation media, salinity, light intensity) on the growth of locally isolated *K. mikimotoi* strain were determined. An optimum growth of *K. mikimotoi* was observed in L1 medium at salinity of 30 ppt with light illuminated at 50-80 μmol photons $m^{-2}s^{-1}$.

Objective 3: Growth of the *K. mikimotoi* stain in standardized L1 algal medium and 30 ppt salinity over 21 days was determined and the growth curves were generated. The Hong Kong strain demonstrated a moderate growth rate of 0.169 day^{-1} when compared to other strains reported in the literature (ranged from 0.126 to 0.336 day^{-1}). The cell density of KMHK increased slowly in the first four days, then growing rapidly from Day 5 onwards and peaked with a density of 2.5×10^4 cells mL^{-1} on Day 13. According to two-way ANOVA analysis, algal cell densities of KMHK ($F_{8, 30} = 193$, $p < 0.05$) were significantly affected by and growth phase. The cell densities in stationary growth phase (2.5×10^4 cells mL^{-1}) were higher than that in log phase (1.9×10^4 cells mL^{-1}) and lag phase (5×10^3 cells mL^{-1}), with cell densities in log phase higher than that in lag phase.

Objective 4: The measure of LT50 was performed by using the *in vivo* medaka test. when the marine medaka *O. latipes* was exposed to *K. mikimotoi* at 2.5×10^4 cells/mL, 100% mortality of the test medaka fish was attained within 60 min. The results reported here showed the LT50 of Hong Kong strain of *K. mikimotoi* on marine medaka was about 30 min. In addition, viability assay on fish gill cells RTgill-W1 after exposed to *K. mikimotoi* cells throughout 120 mintues were conducted. The LT50 of gill cell line exposed to KMHK was 66.9 minutes. Statistical analysis showed that changes in gill cell viability were growth phase dependence ($F_{6, 24} = 33.1$, $p < 0.05$). KMHK at stationary phase were significantly more toxic to gill cells than that at log phase and lag phase.

Objective 5 – 7: Proteomic analysis was performed and experimental conditions for the analysis of dinoflagellate and fish / gill cell samples were optimized. We analyzed the proteomes of different samples over the time-course of exposure. Around 19 key differentially expressed proteins with at-least two-fold expression changes were identified. These proteins are mostly related to inflammatory and oxidative stress responses.

5.4 Summary of objectives addressed to date

Objectives *(as per 5.1/5.2 above)*	**Addressed** *(please tick)*	**Percentage Achieved** *(please estimate)*
1. ***analyse*** the phylogenetic relationship between the locally isolated *Karenia mikimotoi* strain and other strains originating from different geographical locations	✓	100%
2. ***optimise*** the growing conditions of the cell culture of *K. mikimotoi*	✓	100%
3. ***evaluat****e* the effects of growth phases on the ichthyotoxicity of *K. mikimotoi*	✓	100%
4. ***determine*** the median lethal time (LT50) of the ichthyotoxicity of *K. mikimotoi* on the viability of both *in vitro* fish gill cells and *in vivo* medaka fish	✓	100%
5. ***compare*** and ***analyse*** the protein expression profiles of fish gill cells (*in vitro* and *in vivo*) upon exposure to *K. mikimotoi* in terms of different time of exposure and algal cell concentrations as guided by the experiments performed in objectives #3 and 4	✓	100%
6. ***identify*** differentially expressed proteins in different comparative conditions performed in objective #5, and ***validate*** the differential protein expressions using immunoblotting and/or real-real-time PCR techniques	✓	100%
7. ***elucidate*** the possible molecular pathways by which fish gill cells are exposed to *K. mikimotoi*	✓	100%

6. Research Outcome

6.1 Major findings and research outcome
(Maximum 1 page; please make reference to Part C where necessary)

Major findings of this research can be summarized into three aspects: (1) Phylogenetic analysis and growing conditions of a local *K. mikimotoi* strain; (2) Growth and ichthyotoxicity of the strain and (3) Molecular study through proteomic approach.

Phylogenetic analysis and growing conditions of a local *K. mikimotoi* strain
This was the first formal molecular characterization of a *K. mikimotoi* strain (KMHK) isolated from a massive bloom happened in 2016 in Hong Kong. It was found that the Hong Kong strain was closely affiliated to a strain isolated from South China Sea. It provided valuable insight on the possible origin of the Hong Kong strain. The optimized growing conditions for this strain were important for future study on other *K. mikimotoi* strains isolated from Hong Kong and the South China Sea.

Growth and ichthyotoxicity of the strain
We documented the first time of the growth and ichthyotoxicity of the Hong Kong *K. mikimotoi* strain. We found that both growth and toxicity of the strain were highly dependence on the growth phase. The strain achieved the highest growth cell density and toxicity when growing at stationary growth phase. When compared to other *K. mikimotoi* strains, we found that the Hong Kong strain was highly sensitive to the changes of salinity. The optimal salinity for the cells was 30 ppt and they could not survive at salinity of 25 ppt or below. Swelling of cells was observed when the cells growing at 35 ppt. The cell density of KMHK had dropped significantly (more than 80%) even when salinity had changed from 30 ppt to 28 ppt. This may be one of the reasons to explain why blooms of *K.mikimotoi* frequently occurred in Tolo Habour of Hong Kong, where their average salinity ranged from 30 to 34 ppt.

Molecular study through proteomic approach
This was the first report to explore the fish-killing mechanism of *K. mikimotoi* using proteomic approach. We successfully optimized the experimental conditions and established the protocols for the proteomic analysis of the dinoflagellate and fish samples. We identified some key differentially expressed proteins closely related to inflammatory and oxidative stress responses which might be important for the fish-killing actions by *K. mikimotoi*. The established protocols and data generated by this proteomic study would lay a solid foundation for future molecular study on harmful algal samples.

The above findings led to the generation of 7 publications, including 5 SCI journal papers and 2 conference posters.

6.2 Potential for further development of the research and the proposed course of action *(Maximum half a page)*

Build on the results obtained in this study, two possible research directions could be further developed.

Proteomic study on different fish organs and other marine organisms (e.g bivalves) after exposed to *K. mikimotoi*
Experimental conditions for proteomic study on dinoflagellate, medaka fish and fish gill cells have been well established. Apart from the fish gills, proteome changes in different organs of fish or other marine organisms can be examined in order to study the organ-

specific responses and find out the most affected parts of the body by the dinoflagellate. In addition, the exposure study can be further extended to different *K. mikimotoi* strains isolated from different geographic locations.

Development of molecular biosensors for aquatic environmental monitoring

Through the proteomic analysis in this study, several potential protein biomarkers have been identified. To cope with advance computational and electronic technologies, different type of molecular biosensors can be developed. These biosensors can be used for routine monitoring on the health status of the aquatic organisms as well as their associated environmental conditions.

7. Layman's Summary

(Describe in layman's language the nature, significance and value of the research project, in no more than 200 words)

Dinoflagellates are one of the marine microorganisms to cause harmful algal blooms. *Karenia mikimotoi* is a dinoflagellate species and algal blooms caused by them are often associated with massive fish kills around the world. Hong Kong and Mainland China have suffered greatly from algal blooms caused by *K. mikimotoi*. For example, 200 tons of fish were killed by the algal blooms of this species found in Hong Kong Tolo Harbour in 2016. In 2012, algal blooms of this species in Fujian led to massive kill of abalone and resulted in an economic loss of 300 million in US dollars. Our understanding on this deadly algal species is very limited. It remains unclear why and how this species killing fish when they cause algal blooms. Information on the molecular responses of fish to exposure to *K. mikimotoi* is virtually unknown. This research study has formally confirmed the taxonomic identification of a Hong Kong *K. mikimotoi* strain using phylogenetic approach and documented the first time of its cell growth and ichthyotoxicity. We explored the possible molecular fish -killing mechanisms using proteomic approach. Data of this study provided valuable insights and allowed us to delineate the corresponding toxic mechanisms at the molecular level.

Part C: Research Output

8. Peer-Reviewed Journal Publication(s) Arising Directly From This Research Project

(Please attach a copy of the publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.)

The Latest Status of Publications				**Author(s)** *(denote the corresponding author with an asterisk*)*	**Title and Journal / Book** *(with the volume, pages and other necessary publishing details specified)*	**Submitted to RGC** *(indicate the year ending of the relevant progress report)*	**Attached to this Report** *(Yes or No)*	**Acknowledged the Support of RGC** *(Yes or No)*	**Accessible from the Institutional Repository** *(Yes or No)*
Year of Publication	**Year of Acceptance** *(For paper accepted but not yet published)*	**Under Review**	**Under Preparation** *(optional)*						
May 2020				Celia Sze-Nga Kwok, Kaze King-Yip Lai, Sai-Wo Lam, Kin-Ka Chan, Steven Jing-Liang Xu, **Fred Wang-Fat Lee***	Production of high-quality two-dimensional gel electrophoresis profile for marine medaka samples by using Trizol-based protein extraction approaches. *Proteome Science 18*(5), 1-13	**Yes** (manuscript, in mid-term progress report, 1 Jan 2019 – 30 Sep 2019)	**Yes [Attachment 1]**	**Yes**	**Yes**
May 2020				Thomas Chun-Hung Lee, Kaze King-Yip Lai, Celia Sze-Nga Kwok, Steven Jing-Liang Xu, **Fred Wang-Fat Lee***	Comparison of Five TRIzol-Based Protein Preparation Methods for 2-DE Production From Challenging Marine Dinoflagellate Samples: A Case Study on Two Benthic Prorocentrum Species. *Journal of Marine Science and Engineering 8*(5), 363	**No**	**Yes [Attachment 2]**	**Yes**	**Yes**
Jan 2021				Thomas Chun-Hung Lee, Ping-Lung Chan, Nora Fung-Yee Tam, Steven Jing-Liang Xu, **Fred Wang-Fat Lee***	Establish Axenic Cultures of Armored and Unarmored Marine Dinoflagellate Species Using Density Separation, Antibacterial Treatments and Stepwise Dilution	**No**	**Yes [Attachment 3]**	**Yes**	**Yes**

					Selection. *Scientific Reports 11*, 202				
Sep 2021				Kin-Ka Chan, Nora Fung-Yee Tam, Christie Ng, Celia Sze-Nga Kwok, Steven Jing-Liang Xu, Eric Tung-Po Sze, **Fred Wang-Fat Lee***	Proteome Response of Meretrix Bivalves Hepatopancreas Exposed to Paralytic Shellfish Toxins Producing Dinoflagellate Gymnodinium catenatum. *Journal of Marine Science and Engineering 9*(9),1039	**No**	**Yes [Attachment 4]**	**Yes**	**Yes**
Oct 2021				Celia Sze-Nga Kwok, Kaze King-Yip Lai, Winnie Lam, Steven Jing-Liang Xu, Sai-Wo Lam, **Fred Wang-Fat Lee***	Proteome Analysis of Whole-Body Responses in Medaka Experimentally Exposed to Fish-Killing Dinoflagellate Karenia mikimotoi. *International Journal of Molecular Sciences 22* (21), 11625	**No**	**Yes [Attachment 5]**	**Yes**	**Yes**

9. Recognized International Conference(s) In Which Paper(s) Related To This Research Project Was / Were Delivered

(Please attach a copy of each conference abstract)

Month / Year / Place	**Title**	**Conference Name**	**Submitted to RGC** *(indicate the year ending of the relevant progress report)*	**Attached to this Report** *(Yes or No)*	**Acknowledged the Support of RGC** *(Yes or No)*	**Accessible from the Institutional Repository** *(Yes or No)*
June/2019 / Hong Kong	Growth and Toxicity of *Karenia mikimotoi* Strain Isolated From Hong Kong Waters	9th International Conference on Marine Pollution and Ecotoxicology	Yes (in mid-term progress report, 1 Jan 2019 – 30 Sep 2019)	Yes [Attachment 6]	Yes	Yes
June/2019 / Hong Kong	Algicidal Activity of Two Bacterial Strains Isolated from Karenia Blooming Water Against *K. mikimotoi*	9th International Conference on Marine Pollution and Ecotoxicology	Yes (in mid-term progress report, 1 Jan 2019 – 30 Sep 2019)	Yes [Attachment 7]	Yes	Yes

10. Whether Research Experience And New Knowledge Has Been Transferred / Has Contributed To Teaching And Learning

(Please elaborate)

Final Year Projects:

One associated final year project under my supervision was generated. The title of the project was "Critical review on the control of harmful algal blooms". A group of four year 4 (final year year) undergraduate students were working in this project in the period between September 2020 to May 2021. A copy of the title page of the final year project thesis is attached in Attachment 8.

As a case study for undergraduates:

Relevant techniques and results of the study, such as proteomic analysis of harmful algal bloom species, were adopted as case study to share with 150 year 3 students in the course named "Biochemical & DNA Technologies".

STEM workshop:

We organized a relevant train the trainer STEM workshop for secondary school teachers. It was a half-day workshop (9:00 – 14:00) organized on 21 August 2020. Around 10 secondary school teachers attended the workshop. The teachers were trained with both theory and practical skills in microalgal research, such as the isolation, cultivation, growth, toxicity of microalgae [See Attachment 9]

11. Student(s) Trained

(Please attach a copy of the title page of the thesis)

Name	Degree Registered for	Date of Registration	Date of Thesis Submission / Graduation
[redacted]	[redacted]	[redacted]	[redacted]

12. Other Impact

(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, teaching enhancement, etc.)

N.A

13. Statistics on Research Outputs

	Peer-reviewed Journal Publications	Conference Papers	Scholarly Books, Monographs and Chapters	Patents Awarded	Other Research Outputs (please specify)	
No. of outputs arising directly from this research project	5	2	N.A	N.A	Type	No.
					Final year project	1
					STEM module / workshop	1

14. Public Access Of Completion Report

(Please specify the information, if any, that cannot be provided for public access and give the reasons.)

Information that Cannot Be Provided for Public Access	Reasons
N.A	N.A