RGC Ref. No.:
UGC/FDS16/M06/20
(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

Mechanism Underlying Algicidal Activity of P4, a Novel Bacterium Isolated from an Algal Bloom in Hong Kong, against 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)	CHAN Ping-lung / Assistant Professor	School of Science and Technology / Hong Kong Metropolitan University

3. Project Duration

	Original	**Revised**	**Date of RGC / Institution Approval** *(must be quoted)*
Project Start Date	1 January 2021	1 January 2021	
Project Completion Date	31 December 2022	30 June 2023	22 March 2022
Duration *(in month)*	24	30	22 March 2022
Deadline for Submission of Completion Report	31 December 2023	30 June 2024	22 March 2022

4.4 Please attach photo(s) of acknowledgement of RGC-funded facilities / equipment.
N/A

Part B: The Final Report

5. Project Objectives

5.1 Objectives as per original application

1. *determine* the effects of cell concentrations and growth phases of P4 and *K. mikimotoi* on the algicidal activity;
2. *investigate* physiological, biochemical and cell cycle responses of *K. mikimotoi* upon the exposure to P4;
3. *compare* and *analyse* protein expression profiles of both *K. mikimotoi* and P4 cells through their interactions during the algicidal process in terms of (a) different time of exposure, (b) bacterial/algal cell concentrations, and (c) growth phases of bacterial cells and algal cells, as guided by the experiments performed in objective #1;
4. *identify* differentially expressed proteins in various comparative proteomic analyses in objective #3;
5. *elucidate* possible pathways by which *K. mikimotoi* is exposed to P4 and formulate a strategic plan for control of HAB based on the experimental results.

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 successfully completed without any significant issues. The realization of the objectives is summarized below.

Objective 1:
We first conducted literature review to obtain update information and optimized our experimental conditions, such as the optimal cultivation conditions for both the bacterial (P4) and algal (a local *K. mikimotoi* strain, KMHK) cultures. We then carried out a background study to characterize the phylogeny, growth, morphology, gram staining and biochemical / enzymatic properties (for P4) and toxicity (for KMHK) of both the P4 and *K. mikimotoi* cells. For comparison purposes, two additional *K. mikmotoi* strains (a

Japanese strain NIES2411 and a New Zealand strain CAWD133) were included in the experiments alongside the Hong Kong strain. Building on the data from the background study, we investigated the algicidal efficacy of P4 on KMHK under various conditions, including cell concentrations (5, 10, 15, 20, 25 % v/v), algicidal modes (bacterial cell pellet, bacterial supernatant and bacterial cell culture), and growth phases (various growth phases of P4 vs KMHK). We also evaluated the algicidal specificity of P4 to other algal strains, including *Karenia mikimotoi*, *Karenia brevis*, *Prorocentrum triestinum*, and *Alexandrium tamarense*. Finally, we examined the effects of algal axenicity on the algicidal efficacy of P4 by comparing the algicidal effect of P4 on xenic and axenic KMHK cultures.

Objective 2:

Following the results of the above experiments, we adopted the conditions (such as cell density, growth phase) that exhibited the best algicidal effect for subsequent experiments to evaluate the physiological and biochemical changes of the *K. mikimotoi* cells upon P4 exposure. We measured the morphological changes, functionality of the photosystem (in terms of chlorophyll a content and the quantum yield of PSII), oxidative stress (ROS and MDA levels), and cell cycle analysis of the *K. mikimotoi* cells at various time points during the algicidal process.

Objectives 3-5:

Guided by the results obtained from the experiments conducted in Objectives 1-2, we conducted a comparative proteomic analysis in the final part of this project, which aimed to investigate the molecular interaction between P4 and KMHK. Protein expression profiles of both P4 and KMHK cells were analyzed from samples collected under various co-culturing conditions (KMHK + P4 culture, KMHK + P4 supernatant, P4 culture + algal medium as control and KMHK + bacterial medium as control) and times (0 hr, 8 hr and 24 hr). Samples were analyzed through the tandem mass tags (TMT) labeling technique and the Nano LC-Q Orbitrap MS. Both algal and bacterial proteome data were processed using Proteome Discoverer and searched against the available Uniprot proteome databases. Differential expressed proteins (DEPs) were defined if the Log2 fold changes (FC) were ≥ 0.26 (upregulated) or ≤ -0.26 (downregulated) with the p-value ≤ 0.05. The DEPs were then analyzed through the functional annotation and classification using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG).

5.4 Summary of objectives addressed to date

Objectives *(as per 5.1/5.2 above)*	**Addressed** *(please tick)*	**Percentage Achieved** *(please estimate)*
1. *Determine* the effects of cell concentrations and growth phases of P4 and *K. mikimotoi* on the algicidal activity;	✓	100%
2. *Investigate* physiological, biochemical and cell cycle responses of *K. mikimotoi* upon the exposure to P4;	✓	100%
3. *Compare* and *analyse* protein expression profiles of both *K. mikimotoi* and P4 cells through their interactions during the algicidal process in terms of (a) different time of exposure, (b) bacterial/algal cell concentrations, and (c) growth phases of bacterial cells and algal cells, as guided by the experiments	✓	100%

performed in objective #1;		
4. *Identify* differentially expressed proteins in various comparative proteomic analyses in objective #3;	✓	100%
5. *Elucidate* possible pathways by which *K. mikimotoi* is exposed to P4 and *formulate* a strategic plan for control of HAB based on the experimental results.	✓	100%

Research Outcome

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

Characterization on the Karenia mikimotoi and P4: This is the first study to report an algicidal bacterium (P4) that coexists with *K. mikimotoi* (KMHK) bloom in Hong Kong. P4 is a rod-shaped, gram-negative bacterium. Based on the result of 16S rDNA sequencing and the phylogenetic analysis, P4 shared 99.71% similarity with the *M. dokdonensis* strain. The specific growth rates of P4 and KMHK were 0.158 and 0.169 respectively, and the maximum cell density of KMHK was 2.53×10^4 cells mL^{-1}. KMHK was found to be highly ichthyotoxic. Interestingly, we observed that growth and ichthyotoxicity of KMHK were highly sensitive to changes in growth phase and salinity. For the biochemical and enzymatic properties of P4, we found that P4 could produce trypsin and chymotrypsin, suggesting that these enzymes might participate in killing the *K. mikimotoi* cells and therefore terminating the algal bloom. This is supported by other studies that have reported that proteases released by algicidal bacteria may play a significant role in the lysis of algal cells. Part of this data has been published in the Journal of Marine Science and Engineering (Annex 1).

Algicidal effects of P4 on K. mikimotoi: P4 has shown very strong algicidal effect on KMHK. The algicidal effect of P4 culture was not detectable at 5% v/v but was significant at 10% v/v, with the highest value (nearly 100%) observed at 25% v/v dose with 24 hours of exposure. At the highest dose, P4-stressed KMHK cells became rounded, colonized, and rotated. The algal cells gradually lost their motility (at 8 hours) and eventually burst (at 24 hours). The algicidal mode of P4 towards KMHK was found to be indirect. Both the P4 culture and supernatant demonstrated significant algicidal effects, and it was found to be growth phase-dependent. The highest algicidal effect of P4 was observed when both algal and bacterial cells were at stationary phases. This peak efficacy can be attributed to the enhanced algicidal effect of P4 at the stationary phase and the low tolerance of stationary phase KMHK cells. Significant reductions in chlorophyll a content and the Fv/Fm value, and the increase of ROS and MDA levels were observed in KMHK when exposed to P4, suggesting potential damage to the algal photosystem due to the oxidative stress from the bacteria. On the other hand, we found that P4 demonstrated a strong algicidal effect on other dinoflagellate species, indicating its versatility and potential to be used as a universal algicidal bacterium for the removal of algal blooms. Interestingly, we also discovered that the algicidal effect of P4 was significantly higher (> 50%) in xenic than in axenic KMHK, suggesting that there are interactions between KMHK-associated bacteria and P4, which boosts the ability of P4 to kill the algal cells. These findings led to the generation of a publication in the Journal of Phycology (Annex 2) and three conference publications (Annex 4-6).

Molecular study: This was the first study revealing the interactions between harmful algae and algicidal bacteria using proteomics. The proteomic results showed that essential metabolic processes of KMHK cells started to disrupt at 8 hours of co-culture with P4 and such disruption continued until the end of the 24-hour experiment, regardless of co-culture conditions. The disturbance of oxidative phosphorylation in mitochondria and the electron transport chain in chloroplast raised the oxidative stress, leading to the death of KMHK cells. We also found that the iron complex outer-membrane receptor protein in P4 was upregulated after co-culture with KMHK, implying P4 would secrete ferric siderophores, a possible algicidal substance. Furthermore, several stress response proteins of P4 were downregulated at the end of 24-hour co-culture, indicating that P4 was also affected by KMHK. This data led to one publication in the international journal (Annex 3) and one in an international conference (Annex 7).

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

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

Metabolomic study on the cell-to-cell interactions between P4 and KMHK

The experimental conditions for a proteomic study on the algicidal effects of P4 have been well established. The proteomic data from the current study suggest that molecular interactions between P4 and KMHK exist. A metabolomic study could help us understand their cell-to-cell interactions by revealing the exchange and flow of exo- and endo-metabolites between them. This metabolomic study will aid in unraveling the molecular interplay between the two types of bacteria and KMHK, and their modulation of algicidal efficacy.

Study the effect of algal-associated bacteria on the algicidal effects of P4

Our prior experiments have demonstrated that there the algicidal efficacy of P4 could be enhanced by KMHK-associated bacteria. This finding clearly indicated that algicidal bacteria interact not only with their target algae but also with the algal-associated bacteria, significantly affecting the efficacy of the algicidal bacteria. Therefore, it is crucial to understand the roles of the bacteria associated with the algae and the mechanism of their influence on the efficacy of algae killing, as this closely reflects the natural environmental conditions.

6. Layman's Summary

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

Harmful algal bloom (HAB) is a big environmental problem around the world. It damages the fish farming and shellfish industries and can be dangerous to people's health. Mainland China and Hong Kong have been severely affected by HAB. Over the years, various methods have been employed to control HABs. However, these methods are either expensive or can cause secondary pollution. Our research focused on a type of bacteria that could be a cheaper and more sustainable way to control HABs. We studied a specific type of bacteria (algicidal bacteria P4) that kills algae (*Karenia mikimotoi*, KMHK), both found in Hong Kong. *K. mikimotoi* is a well-known toxic HAB species with very powerful fish-killing ability. We found that P4 can kill KMHK by secreting algicidal substances. This effect depends on how much bacteria is present, the growth stage of the algae and bacteria, and the presence / absence of algal-associated bacteria. We also studied how the algae and bacteria interact on a molecular level using proteomic approach. Our findings provide valuable insights into the interaction between algae and bacteria, which aid us in developing effective strategies for the control of HABs.

Part C: Research Output

7. 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 correspond-ing 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)*	**Acknowl-edged 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)*						
2022				Winnie Lam, Emily Man-Shan Cheung, Nora Fung-Yee Tam, Thomas Chun- Hung Lee, Celia Sze-Nga Kwok, Kaze King-Yip Lai, Steven Jingliang Xu, **Fred Wang-Fat Lee ***	Effects of salinity on growth and in vitro ichthyotoxicity of three strains of *Karenia mikimotoi.* *Journal of Marine Science and Engineering 10(9):1236*	No	Yes [Annex 1]	Yes	Yes
2024				Thomas Chun-Hung Lee, Winnie Lam, Nora Fung-Yee Tam, Steven Jing-Liang Xu, Wing Lam Chung, **Fred Wang-Fat Lee***	Revealing the algicidal characteristics of *Maribacter dokdonensis*: An investigation into bacterial strain P4 isolated from *Karenia mikimotoi* bloom water. *Journal of Phycology, 60(2): 541-553*	No	Yes [Annex 2]	Yes	Yes
Submitted in June 2024		**Yes**		Thomas Chun-Hung Lee, Winnie Lam, Nora Fung-Yee Tam, Ichiro Imai, Steven Jing-Liang Xu, Ping-Lung Chan, **Fred Wang-Fat Lee***	Proteomic insights of interaction between ichthytoxic dinoflagellate *Karenia mikimotoi* and algicidal bacteria *Maribacter dokdonensis* P4	No	Yes [Manuscript is attached. Please see Annex 3]	Yes	Yes

					The manuscript has been submitted to *Science of the Total Environment*				

8. 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)*	**Acknowl-edged the Support of RGC** *(Yes or No)*	**Accessible from the Institutional Repository** *(Yes or No)*
January / 2023/ Xiamen, China	Algicidal activity of P4, a bacterial strain isolated from an algal bloom in Hong Kong, against *Karenia mikimotoi*	The Sixth Xiamen Symposium on Marine Environmental Sciences (XMAS)	No	Yes [Annex 4]	Yes	Yes
August / 2023/ Hong Kong	Advancing the Establishment of Bacterial – free *Karenia mikimotoi* Cultures: A promising Methodology and Characterization of an Algicidal Bacterial Strain isolated from a *K. mikimotoi* Bloom in Hong Kong	International conference on algal research, application and management	No	Yes [Annex 5]	Yes	Yes
November / 2023/ Hiroshima, Japan	Toxicity effect of the algicidal supernatant from bacterium P4 and the *Karenia mikimotoi* cells after exposed to algicidal supernatant on fish gill cell line	20th International conference on harmful algae (ICHA)	No	Yes [Annex 6]	Yes	Yes
January / 2024 / Hong Kong	Proteomic insights of the interaction between ichthyotoxic dinoflagellate *Karenia mikimotoi* and algicidal bacteria *Maribacter dokdonensis*	10th International conference on marine pollution and ecotoxicology	No	Yes [Annex 7]	Yes	Yes

9. Whether Research Experience And New Knowledge Has Been Transferred / Has Contributed To Teaching And Learning
(Please elaborate)

Final Year Project:
I supervised a final year project titled "An Overview of Harmful Algal Blooms: Algal Toxins, Bacterial Communities, and Global Impacts". This project was undertaken by a group of four final year undergraduate students from September 2021 to May 2022. A copy of the title page of the final year project thesis is attached in Annex 8.

STEM workshop:
We organized two relevant STEM workshops for secondary school teachers and students. The participants were trained in both theoretical and practical aspects of microalgal research, including the isolation, cultivation, growth, and toxicity of microalgae. The two workshops were held on 5 August 2021 (with 40 participants) and 18 June 2022 (with 30 participants) respectively. [See Annex 9]

As a case study for undergraduates:
Relevant techniques and results from the study, such as proteomic analysis of harmful algal bloom species, were adopted as case study and shared with around 120 year 3 students in the course titled “Biochemical & DNA Technologies”.

10. 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
N/A	N/A	N/A	N/A

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

I was invited by the organizing committee to serve as a speaker at the 10th International Conference on Marine Pollution and Ecotoxicology in January 2024. The title of my talk was *"Possible Role of Marine Bacteria in Modulating Harmful Algal Blooms of Karenia mikimotoi*" [Annex 10].

12. 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	2 [published] + 1 [under review]	4	N/A	N/A	Type	No.
					Trained Final year project students	4
					STEM training workshop	2
					As invited speaker to give a talk in an international conference	1

13. 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