Darwin Initiative for the Survival of Species

Annual Report (2000-2001)

BACTERIAL DIVERSITY IN COASTAL SEAWATER IN SHANDONG PROVINCE, CHINA

DETR 162/8/170

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Annual Report

1. Darwin Project Information

Project title	Bacterial diversity in coastal seawater in Shandong Province, China
Country(ies)	U.K. and P.R. China
Contractor	Professor B. Austin (Heriot-Watt University)
Project Reference No.	DETR 162/8/170
Grant Value	£123,688
Start/Finishing dates	April 1 1999-March 31, 2002
Reporting period	Year 2 (2000-2001)

2. Project Background

- The project involves co-operation with the Ocean University of Qingdao [OUQ] (Qingdao, Shandong, P.R. China), and seeks to examine the bacterial diversity in coastal seawater in the vicinity of Qingdao, and relate the diversity to industrial and aquacultural pollution. Experimental sites are based within easy reach of Qingdao

3. Project Objectives

- To establish a unit within the OUQ to specialise in the development of methods for the study of bacterial biodiversity in coastal seawater habitats
- To initiate a programme of data collection on biological, physical and chemical parameters at polluted and clean coastal sites in Shandong Province
- To train Chinese researchers during training courses in the UK and in Qingdao on the techniques and methodology relevant for bacterial isolation and identification
- To assist OUQ in promoting its activities of increasing awareness of fragility of Shandong's coastal habitats and the need for sustainable management of them among local residents, industry and visitors
- To assist OUQ in attracting further research and development funding
- To extend and develop Heriot-Watt University's staff and research student expertise in marine microbiological problems in China.

- The Objectives have not been modified during the current year.

4. Progress

- Following an initial planning meeting in Edinburgh (Professor H.-S. Xu and Li Yun attended), suitable sites in the vicinity of Qingdao were identified, and a detailed sampling programme was designed. Initially, a trial sampling session was carried out. Then at approximately two monthly intervals, the mainstream research programme was carried out. Essentially, physico-chemical parameters were measured, and bacterial populations examined (both quantitatively and qualitatively). Training has been carried out in both Qingdao and Edinburgh, and there has been an exchange of staff regularly throughout the two years of the project.

- Professors Austin and Priest and/or Dr. Robertson have visited OUQ on three occasions during the last year. The two Darwin Scholars (De-Hua Yu and Zong-Jun Du) visited Edinburgh for a training period studying bacterial identification and taxonomy (formal diplomas were presented to the two Darwin Fellows at the end of the course) and have since been instrumental in training others at OUQ. Thus, during Year 2 a total of 4 staff, 4 graduate students and 2 Ph.D. students were involved in the project at OUQ. The initial stages of the research programme have been completed, and the taxonomic stages have started and, indeed are mostly finished. Thus to date, the data for the Gram-negative bacteria have been completed and analysed. The Gram-positive bacteria are currently in the final stages of examination. A start has been made on the molecular aspects of the project, and have involved staff from the Centre for Marine Biodiversity and Biotechnology (also at Heriot-Watt University). Two talks have been given at conferences, and two manuscripts prepared for publication. The Workshop, scheduled for July 2001, has been largely planned.

- The detailed research programme designed during an initial meeting in Edinburgh (during Year 1) was subsequently refined after a trial sampling experiment and finalised as a result of a meeting in OUQ. The plan has been to examine coastal sites in the vicinity of Qingdao. These sites include a control (unpolluted site), a site exposed to pollution from a coal-fired power station and dyehouse effluent, and sites around fish/shellfish farms. These sites were sampled on a two-monthly basis, with physico-chemical data (temperature, salinity, turbidity, pH and nitrate levels were recorded) and bacteriological data (quantitative by microscopy and spread plates to obtain colonies) collected from water, sediment, seaweed and aquatic animals (fish, scallops). A random collection of isolates was purified, and examined by basic techniques, essentially to divide the population into Gram-negative and Gram-positive components. Already, the dominant Gram-negative component has been examined by BIOLOG-GN, and the data clustered using NTSYS (a synopsis of the output is presented in the Appendix). The Gram-positive bacteria are currently being examined for cell wall components and 16S and 23S rRNA gene sequences. Initial work has been carried out to extend the molecular approach to target specified vibrios, notably *Vibrio harveyi, V. parahaemolyticus* and *V. campbellii.* In addition, in collaboration with researchers from the Centre for Marine Biodiversity and Biotechnology, the culture collection is currently being screened for production of bioactive molecules, which may have potential for biotechnology applications. Training and advise in marine microbiology was given during visits by Professors Austin and Priest and Dr. Robertson to OUQ and during a formal training period in Edinburgh. Thus, the two Darwin Scholars (De-Hua Yu and Zong-Jun Du) visited Edinburgh for training in general identification techniques relevant to marine bacteria and specifically with BIOLOG-GN. The Scholars, chosen by Professor Xu, worked with isolates brought from OUQ, and took part in clustering and analyses of the

data. In addition, they participated in developing molecular probes for *V. harveyi, V. parahaemolyticus* and *V. campbellii*. These probes will be used in further field work in Qingdao. An initial paper was presented at the 2nd International Symposium of Marine Biotechnology in December 2000.

- There have not been any significant difficulties experienced over the last year. Indeed, the co-operation has progressed extremely well.
- There have been refinements (= improvements made to the project) during Year 2, as the group learns from its experiences. For example, revisions had been made to the sampling programme. Thus, samples were collected and examined over several days rather than the single day sampling regimes initially perceived. However, there have not been any changes to the original goals of the project.
- Timetable/Work Plan for Year 3.

July	Workshop and final steering meeting to be held at OUQ (The key papers will be published in a special issue of the Journal of the Ocean University of Qingdao)
September	Completion of examination of Gram-positive bacteria
December	Completion of analyses (regarding biodiversity)
February	Completion of molecular based study
March	Preparation of at least three scientific manuscripts
March	Preparation of final report

5. Partnerships

- The collaboration between Edinburgh and Qingdao continues to be most fruitful, with regular communication by means of Email. We have supported Qingdao in its (successful) bid to obtain substantive funding from the Chinese Ministry of Science and Technology (600 million Yuan were awarded). The title of Guest Professor of the Ocean University of Qingdao was conferred on Professor Austin. We have assisted Qingdao with applications to UNESCO (for a training programme) and in the organisation of the 2nd International Symposium of Marine Biotechnology.
- There has been collaboration within the P.R. China, with staff from OUQ attending the China-EU Workshop in Biodiversity, which was held at Xishuang Banna in January 2001. Also, there is evidence of collaboration between different groups within OUQ and with organisations in Shandong – such as the Yellow Sea Fisheries Institute. Links, relating to marine microbiology, have been forged with UNESCO, with OUQ delivering a training programme to students from Asian countries.
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6. Impact and Sustainability

- The work has been promoted informally by Professor Xu to the research institutes in Qingdao, and at formal meetings such as the China-EU Workshop on Biodiversity (held in Xishuang Banna in January 2001) and at the 2nd International Symposium of Marine Biotechnology (held in Qingdao in December 2000). Publicity has been generated by fliers, specifically announcing the Workshop, which is scheduled for July 2001. These fliers have been distributed to all relevant Departments and Institutes in the P.R. China. An increasing interest in

the work area can be attested by the large number of queries that are regularly received by Email. In terms of exit strategies, the OUQ is now well positioned to develop the work area by means of moneys from the Chinese Ministry of Science and Technology and UNESCO. However, the project has identified an interesting area that OUQ would benefit from further co-operation. I refer to the so-called unculturable bacteria, which are in evidence but require detailed work beyond the scope of the current project.

7. Outputs, Outcomes and Dissemination

Table 1. Project Outputs (According to Standard Output Measures)

Code No.	Quantity	Description
6A/B	2	2 Chinese scientists were trained in rapid identification techniques in the U.K. for 3 weeks

The specified Project Output was completed, when the two Darwin Scholars (De-Hua Yu and Zong-Jun Du) visited Edinburgh for a 3-week plus training period in bacterial identification and taxonomy. Specifically, the Scholars examined isolates from the project, utilising commercially available rapid identification systems, notably BIOLOG-GN, which is used for characterisation of Gram-negative bacteria. The resulting data was clustered by means of the statistical analysis software, NTSYS. The two Scholars also worked on developing molecular probes for three target vibrios, *V. harveyi*, *V. parahaemolyticus* and *V. campbellii*. The technique will be used at OUQ for determining the incidence of these organisms in coastal waters. Diplomas, printed on parchment and incorporating the Darwin Crest, were presented to the two Darwin Fellows at a formal dinner held at the end of the course.

- We have carried out all the contractual duties for Year 2. In addition, we have extended the research activities and already produced publications and presented data at meetings. Heriot-Watt University was able to visit OUQ on three occasions, of which one was financed by the Chinese Ministry of Science and Technology.

Table 2: Publications

Type *	Detail	Publishers	Available from	*Cost £*
Book	Proceedings of the 2nd International Symposium on Marine Biotechnology	Ocean University of Qingdao,	Ocean University of Qingdao	*?*

- Talks were given at The China-EU Workshop on Biodiversity (held in Xishuang Banna in January 2001) and at the 2nd International Symposium of Marine Biotechnology (held in Qingdao in December 2000). We will be planning for the future during the forthcoming year.

8. Project Expenditure

Table 3: Project expenditure during the reporting period

Item	Budget	Expenditure
Total	35662	35662

9. Monitoring, Evaluation and Lessons

- Monitoring and evaluation have been facilitated by regular meetings, Email and regular written reports, with outcomes compared against the original application. Our achievements can be measured against written reports, the information from which is being used to draft scientific papers.
- The lessons are that it is imperative to maintain regular contact with overseas partners and to show great patience. Email has been invaluable for communication. Face-to-face contact is important, and I wish that more visits had been budgeted. Nevertheless, we have been fortunate that funding for extra visits has been secured from The Chinese Ministry of Science and Technology and British industry.

10. Author(s) / Date

Professor B. Austin (with input from Professor H.S. Xu and Dr. P.A.W. Robertson)

APPENDIX

Sampling strategy

- Reason – to explore the impact of pollution
- Sampling sites within easy reach of Qingdao: industrially polluted; aquaculture; unpolluted control
- Frequency of sampling: every two months for one year
- Time of sampling: standardised according to time of day and state of the tide
- Samples: fish; invertebrates; seaweed – for epibionts; water; sediment
- Physico-chemical parameters: temperature; pH; salinity; nitrate
- Microbiological methods
    - Microscopy direct viable count – Kogure's method
    - Culturing: marine 2216E agar; TCBS for vibrios; MacConkey agar for enterics; seaweed extract agar; dilute medium [sea water gelled with agar] for oligotrophs. Diluent – 0.9% (w/v) saline; incubation at ambient temperature for up to 14 days or 28+ days for oligotrophs
    - Cultures: count plates; select random colonies for purification
    - Initial screen - Gram stain; O-F test; catalase; oxidase; sensitivity to 0/129
    - Secondary screen by BiologGN (using cultures sent to HWU)
- Computer based data matrix to be constructed and groups defined and identified.

11. TAXONOMIC STUDY

12. Gram-negative bacteria

A total of 266 strains out of the 335 Gram-negative isolates (= 79%) were equated within 50 clusters when examined using BiologGN

Distribution of clusters between sites:

Site XLZ contained representatives of 38 clusters

Site PS contained representatives of 22 clusters

Site ELS contained representatives of 6 clusters

Site 10 contained representatives of 6 clusters

Site 8 contained representatives of 3 clusters

Site CJB contained representatives of 3 clusters

Site LST contained representatives of 2 clusters

Distribution of clusters according to sampling month:

February	representatives of 9 clusters
April	representatives of 17 clusters
June	representatives of 7 clusters
August	representatives of 13 clusters
October	representatives of 30 clusters
December	representatives of 12 clusters

Distribution of clusters according to sample type:

SED	representatives of 27 clusters
SWD	representatives of 16 clusters
SW	representatives of 30 clusters
F/SW	representatives of 5 clusters
Fish	representatives of 16 clusters
Scallop	representatives of 1 cluster
Shrimp	representatives of 1 cluster

Specificity of distribution:

Isolates from 25 clusters were restricted to a single sampling month

Isolates from 22 clusters were restricted to single locations

Isolates from 16 clusters were restricted to single sample types

Sediment only clusters: 5, 6, 11, 14, 31, 36 and 50

Seawater only clusters: 4, 7, 8, 10, 37 and 49

Seaweed only clusters: 21

Fish only clusters: 19, 28 and 33

Shrimp only clusters: 1

Distribution of unclustered isolates between sampling site

Site	Sample type	No profile	Profiled	Total
XLZ	SED	17	14	31
	SWD	12	12	24
	SW	22	10	32
	Scallop (healthy)	2	0	2
	Fish (healthy)	4	6	10
	Fish (diseased)	1	2	3
	Total	**58**	**44**	**102**
PS	SED	20	5	25

	SW		12	7	19
		Total	**32**	**12**	**44**
8	SED		6	3	9
	SW		3	0	3
		Total	**9**	**3**	**12**
10	SED		1	3	4
	SW		1	1	2
		Total	**2**	**4**	**6**
ELS	SED		**1**	**2**	**3**
LST	SW		**6**	**2**	**8**
CJZB	SED		1	1	2
	SW		0	1	1
		Total	**1**	**2**	**3**
Thailand		SW	**0**	**1**	**1**
Reference strains			**2**	**20**	**22**
Total			**111**	**90**	**201**

Distribution of unclustered isolates according to sample type

Sediment	74
Seawater	66
Seaweed	24
Scallop (healthy)	2
Fish (healthy)	10
Fish (diseased)	3
Reference strains	22
Total	201

Distribution of unclustered isolates according to sampling month

	Unprofiled	Unclustered	Monthly G- isolates (% unclustered)
October 1999	26	15	81 (50.6%)
December 1999	20	6	59 (44.1%)
February 2000	24	11	55 (63.6%)
April 2000	5	7	49 (26.5%)
June 2000	11	7	41 (43.9%)
August 2000	8	7	66 (22.7%)
October 2000	15	17	104 (31.7%)

Total	**109**	**70**	**455 (40.2%)**

13. Gram-positive bacteria

In total 119 Gram-positive isolates were isolated, comprising 97 rods (81.5%), 14 cocci (11.8%), 6 coccobacilli (5%), and 2 with variable morphology (1.7%).

Distribution of isolates between sampling site (% of Gram positive isolates)

XLZ	SED		20
	SWD		19
	SW		13
	Scallop (healthy)		3
	Fish (healthy)		9
	Fish (diseased)		6
		Total	70 (58.8%)
PS	SED		10
	SW		17
		Total	27 (22.7%)
8	SED		5
	SW		2
		Total	7 (5.9%)
10	SED		1 (0.8%)
ELS	SED		6 (5.0%)
LST	SW		7 (5.9%)
CJZB	SW		1 (0.8%)

Distribution of isolates according to sample type

Sediment	42 (35.3%)
Seawater	40 (33.6%)
Seaweed	19 (16.0%)
Scallop (healthy)	3 (2.5%)
Fish (healthy)	9 (7.6%)
Fish (diseased)	6 (5.0%)

Distribution of isolates according to sampling month/total monthly isolates

October 1999	25/106 (23.6%)
December 1999	20/79 (25.3%)
February 2000	14/69 (20.3%)

April 2000	16/65 (24.6%)
June 2000	13/54 (24.1%)
August 2000	11/77 (14.3%)
October 2000	20/124 (16.1%)
Total	119/574 (20.7%)

Abbreviations

XLZ: Xianlangzui (aquaculture site)

PS: power station (industrially polluted site)

LST: Laoshan (unpolluted site)

Site 8: mouth of Jiaozhou bay

Site 10: centre of Jiaozhou bay

ELS: end of Laoshan

SED: sediment

SW: seawater

SWD: seaweed

F/SW: seawater from fish rearing cage