Practical workshop on virus ecology methods

Marine Biological Association (MBA) of the UK, Plymouth

23 - 28 July 2006

Workshop Manual

Welcome statement

Nick Owens

Steve Hawkins

Dear Participants
We are delighted to welcome you to our beautiful city and the world-class research institutes that are located here. Drs Wilson and Schroeder have put together a fantastic workshop and will be ably assisted by other world experts in virus ecology. I am sure this workshop will be a resounding success and I hope it will lead to enduring collaborations between participants and Plymouth based scientists.
Welcome again, we are sure you will benefit not only from the technical programme, but also from your stay in Plymouth – THE place for Marine Science in the UK!
Very best wishes

PROFESSOR NICHOLAS J P OWENS
Director PML

PROFESSOR STEVE HAWKINS
Director MBA

We are grateful for the generous sponsorship and support from the following organisations:

The workshop manual was compiled by *Susie Wharam*.

It should be stressed that this manual is made up of contributions from each of the experts and demonstrators. We are extremely indebted to everyone involved, for the outstanding effort they have put into this project.

Willie Wilson & Declan Schroeder

Programme at a glance

Date in July 2006	Morning				Afternoon				Evening	
	0900 – 1000	Methods overview	Coffee	1100-1230	Lunch	1330-1500	Coffee	1530-1700	1800 Talk & nibbles:	1900 Dinner
Sunday 23	Arrive & Register Opening address				PML From 12 noon PML intro then sampling				Meal on a River Tamar pleasure cruise (1730)	
Monday 24	Case study Curtis Suttle (Heterosigma viruses)			TFF VC (centrifugn) AFC		AFC TFF VC (centrifugn)		VC (centrifugn) AFC TFF	POSTER Session Meal at MBA from 1900	
Tuesday 25	Case study Keizo Nagasaki (Diatom viruses)			TEM Plaque assays Probe Design		Probe Design TEM Plaque assays		Plaque assays Probe Design TEM	Posters John Burden talk Meal at MBA	
Wednesday 26	Eden Project					Case studies: Eric Wom Steven Wil Corina Bru R-A Sandaa Markus Wein		Wine & nibbles	Back to Plymouth & Free Time	
Thursday 27	Case study Jim van Etten (Large ds DNA viruses)			Nuc Ac Extn Productivity QPCR		QPCR Nuc Ac Extn Productivity		Productivity QPCR Nuc Ac Extn	Posters Nick Mann Talk Plymouth Gin Tour	
Friday 28	Case study John Paul (Lysogeny/Latency)			EFM PFGE DGGE		DGGE EFM PFGE		PFGE DGGE EFM	Burns night	

Method code	Description	Experts	Demonstrator
Sampling	Collection of samples from seawater	Steven Wilhelm (USA); Willie Wilson & Susan Kimmance (PML)	Ellie Harrison (PML) Karen Weynberg (PML
TFF	Tangential flow filtration	GE Healthcare	Karen Weynberg (PML)
VC	Concentration of viruses onto electron microscope grids (for analysis during TEM work)	Willie Wilson (PML)	Matt Hall (MBA)
Productivity	Dilution experiment set up	Markus Weinbauer (France)	Susan Kimmance (PML)
AFC	Analytical Flow Cytometry	Corina Brussaard (The Netherlands)	Claire Evans (PML)
Plaque assays	Isolation of viruses by plaque assay	Jim van Etten (USA)	Andrea Baker (MBA)
EFM	Epiflourescence microscopy (virus counting	Curtis Suttle (Canada)	Steve Ripley (MBA)
Nuc Ac Extn	Nucleic acid extraction	John Paul (USA)	Mike Allen (PML)
Probe design	Design of virus specific probes (PC – based exercise?)	Declan Schroeder (MBA)	Nick Bloomer (MBA – IT support)
QPCR	Quantitative PCR	Techne	Karen Weynberg (PML)
TEM	Electron Microscopy	Keizo Nagasaki (Japan)	Matt Hall (MBA); Willie Wilson (PML)
PFGE	Pulsed Field Gel Electophoresis	Eric Wommack (USA)	Jayme Lohr (PML)
DGGE	Denaturing Gradient Gel Electrophoresis	Ruth-Anne Sandaa (Norway) Joaquin Martinez Martinez (The Netherlands)	Declan Schroeder (MBA)

Contents

Detailed Timetable

Sunday 23rd July

Date Time	Activity			Location
0900-1100	Arrive and register			Marine Biological Association (MBA)
1100-1145	**Opening address:** Declan Schroeder Willie Wilson			MBA Resource Centre
1145-1200	Walk to Plymouth Marine Laboratory (PML)			
1200–1400	Barbeque			PML
1400-1430	**Introduction to PML**: Dave Robins, Director of PML Applications			PML Coffee Room
1430	**Sample collection and filtration:** Students split into 3 groups (Red, Green & Blue) 3 x 30 minute sessions **Session 1:** Sampling equipment demonstration (Willie Wilson) **Session 2:** Processing seawater for virus analysis (Steven Wilhelm & Karen Weynberg) **Session 3:** Hands on filtering session (Susan Kimmance & Ellie Harrison)			PML
	Session 1 Mesocosm	**Session 2** Seawater Hall	**Session 3** Room 111	
1430-1500	Red	Green	Blue	
1500-1530	Green	Blue	Red	
1530-1600	Blue	Red	Green	
1700	Meet at the MBA			MBA
1710	Walk to Phoenix Wharf			
1730	Set sail on Booze Cruise! (approx. 3h)			

Notes:

Monday 24th July

Time	Activity			Location
0900 - 1000	Case study: Curtis Suttle **VIRUSES INFECTING *HETEROSIGMA AKASHIWO***			Resource centre
1000–1030	Methods briefings			Resource centre
1030-1100	COFFEE			Mess room
1100	Students split into 3 groups (Red, Green & Blue) **TFF:** Tangential flow filtration (GE Healthcare) **VC:** Virus concentration for TEM (Willie Wilson & Matt Hall) **AFC:** Analytical Flow Cytometry (Corina Brussaard & Claire Evans)			All at the MBA
	TFF Room 5	**VC** Room 82	**AFC** Resource Centre	
1100–1230	Red	Green	Blue	
1230 - 1330	LUNCH			Mess room
1500 - 1530	Green	Blue	Red	
1530 - 1600	COFFEE			Mess room
1530-1700	Blue	Red	Green	
1730 - 1900	POSTER SESSION (Prize for best poster) Wine and nibbles included			Resource Centre
1900 -	DINNER			Mess room

Notes:

Tuesday 25th July

Time	Activity			Location
0900 - 1000	Case study: Keizo Nagasaki **ISOLATION AND CHARACTERISATION OF VIRUSES INFECTING MARINE EUKARYOTIC MICROALGAE**			Resource centre
1000–1030	Methods briefings			Resource centre
1030-1100	COFFEE			Mess room
1100	Students split into 3 groups (Red, Green & Blue) **TEM: Transmission Electron Microscopy** (Jeol; Keizo Nagasaki; Pete Bond; Roy Moate). NB *This session will be conducted at the University of Plymouth Electron Microscopy Centre* (**UoP**) **Plaque Assays:** (Jim van Etten & Andrea Baker) **Probe Design:** (Declan Schroeder)			MBA and University of Plymouth.
	TEM UoP	**Plaque assays** Room 82	**Probe Design** Resource Centre	
1100–1230	Red	Green	Blue	
1230-1330	LUNCH			Mess room
1500 - 1530	Green	Blue	Red	
1530 - 1600	COFFEE			Mess room
1530-1700	Blue	Red	Green	
1700 - 1800	POSTER SESSION (Prize for best poster) Wine and nibbles included			Resource Centre
1800	Guest Speaker: John Burden **INSECT VIRUSES – ECOLOGY, BIOTECHNOLOGY AND MANIPULATION IN THE GARDEN.**			Resource Centre
1900 -	DINNER			Mess room

Notes:

Wednesday 26th July

Time	Activity	Location
0830	Pick up bus for Eden project	MBA
1000	Arrive at Eden project	
1030	Meet at the horse at the top of the visitor centre	
1030 – 1230	Guided tour of the Eden project	
1230 - 1330	Buffet lunch in the core building	Outside rooms 3 & 4
1400	Minibus to Foundation building from core building	
1430	Case studies	Training rooms 1 & 2
1430	Eric Wommack **METAGENOMICS AND THE MARINE VIRUS COMMUNITY**	
1500	Steven Wilhelm **SAMPLING FOR VIRUSES ACROSS OCEANIC REALMS: WHERE ARE YOU GOING AND WHAT ARE YOU DOING?**	
1530	Corina Brussaard ***PHAEOCYSTIS* VIRUSES**	
1600	Ruth-Anne Sandaa **VIRAL COMMUNITY ANALYSIS.**	
1630	Markus Weinbauer **DETERMINATION OF VIRUS-INDUCED MORTALITY IN MARINE SYSTEMS**	
1700	Wine and nibbles	
1745	Bus back to Plymouth	
1900	Arrive Plymouth	
EVENING	**Free time**	

Notes:

Thursday 27th July

Time	Activity			Location
0900 - 1000	Case study: Jim van Etten **CHLORELLA VIRUSES**			Resource centre
1000 – 1030	Methods briefings			Resource centre
1030 - 1100	COFFEE			Mess room
1100	Students split into 3 groups (Red, Green & Blue) **Nuc Ac Extn:** Nucleic Acid Extraction (John Paul & Mike Allen). **Productivity:** Dilution experiments to determine virus productivity (Markus Weinbauer & Susan Kimmance) **QPCR:** Quantitative PCR (Techne)			MBA
	Nuc Ac Extn: Room 5	**Productivity** Resource Centre	**QPCR:** Room 82	
1100 – 1230	Red	Green	Blue	
1230 - 1330	LUNCH			Mess room
1500 - 1530	Green	Blue	Red	
1530 - 1600	COFFEE			Mess room
1530-1700	Blue	Red	Green	
1700 - 1730	POSTER SESSION (Prize for best poster) Wine and nibbles included			Resource Centre
1730	Guest Speaker: Nick Mann **EVOLUTIONARY PRESSURES ON MARINE VIRUSES**			Resource Centre
1900 -	Plymouth Gin Distillery Tour			Plymouth Gin Distillery
2015	DINNER			Plymouth Gin Distillery

Notes:

Friday 28th July

Time	Activity			Location
0900 - 1000	Case study: John Paul **PROPHAGES: DANGEROUS MOLECULAR TIME BOMBS OR THE KEY TO BACTERIAL SURVIVAL IN THE OCEANS?**			Resource centre
1000 – 1030	Methods briefings			Resource centre
1030 - 1100	COFFEE			Mess room
1100	Students split into 3 groups (Red, Green & Blue) **EFM:** Epiflourescence Microscopy for virus counting (Curtis Suttle & Steve Ripley) **PFGE:** Pulsed Field Gel Electrophoresis (Eric Wommack & Jayme Lohr) **DGGE:** Denaturing Gradient Gel electrophoresis (Ruth-Anne Sandaa, Joaquin Martinez Martinez & Declan Schroeder)			MBA
	EFM Room 82	**PFGE** Room 5	**DGGE** Room 76	
1100 – 1230	Red	Green	Blue	
1230 - 1330	LUNCH			Mess room
1500 - 1530	Green	Blue	Red	
1530 - 1600	COFFEE			Mess room
1530-1700	Blue	Red	Green	
1700 - 1900	**GET YER KILTS ON!**			
1900 -	BURNS NIGHT			Mess room

Notes:

Laboratory Protocols

Sunday 23rd July

Date Time	Activity			Location
0900 - 1100	Arrive and register			Marine Biological Association (MBA)
1100 - 1145	**Opening address:** Declan Schroeder Willie Wilson			MBA Resource Centre
1145 - 1200	Walk to Plymouth Marine Laboratory (PML)			
1200 – 1400	Barbeque			PML
1400 - 1430	**Introduction to PML**: Dave Robins, Director of PML Applications			PML Coffee Room
1430	**Sample collection and filtration:** Students split into 3 groups (Red, Green & Blue) 3 x 30 minute sessions **Session 1:** Sampling equipment demonstration (Willie Wilson) **Session 2:** Processing seawater for virus analysis (Steven Wilhelm & Karen Weynberg) **Session 3:** Hands on filtering session (Susan Kimmance & Ellie Harrison)			PML
	Session 1 Mesocosm	**Session 2** Seawater Hall	**Session 3** Room 111	
1430-1500	Red	Green	Blue	
1500-1530	Green	Blue	Red	
1530-1600	Blue	Red	Green	
1700	Meet at the MBA			MBA
1710	Walk to Phoenix Wharf			
1730	Set sail on Booze Cruise! (approx. 3h)			

Notes:

Sunday 23rd July

Sample collection and filtration:

Experts: Steven Wilhelm, Willie Wilson, Susan Kimmance

Location: Plymouth Marine Laboratory

Start: 1430

Students split into 3 groups (Red, Green & Blue)

3 x 30 minute sessions
Session 1: Sampling equipment demonstration (mesocosm)
Session 2: Processing seawater for virus analysis (seawater hall)
Session 3: Hands on filtering session (room 111)

	Session 1	Session 2	Session 3
1430-1500	Red	Green	Blue
1500-1530	Green	Blue	Red
1530-1600	Blue	Red	Green

Purpose: To develop insight into how water samples should be collected to obtain the best possible sample and to demonstrate how the end use of the samples will often dictate how the water is collected.

Introduction: The single most critical part of biological oceanographic research is the proper collection and handling of samples. In the case of viruses, research must couple a level of aseptic technique with the collection of often large (> 200 L) volumes of water and the inherent difficulties of working at sea. As part of this module, the instructors will walk students through these sessions designed to provide some insight into the processes that may commonly be used to collect and pre-process seawater samples for studies with viruses. Information will also be provided on approaches for the designation of sampling codes so that parallel datasets can easily be resolved.
A case study (in this case more of a technical review) will be provided that will discuss the various methods of water sample collection, considerations to be made in collecting samples and results from field studies looking at realistic temporal and spatial variations in virus abundance and activity in order to illustrate the natural range of variations being dealt with. Sample collection approaches involving a variety of sampling vessels (Niskins, Go-Flos, Vandorens, etc.) and other approaches (*e.g.*, underway pumping systems and associated "fish") will be discussed. Information will also be presented on the pros and cons of different filters that can be chosen for the pre-filtration or collection of viruses from marine water samples.

Millipore: http://www.millipore.com/catalogue.nsf/home
Sartorius: http://www.sartorius.com
VWR: http://uk.vwr.com/app/Home
Fisher: http://www.fisher.co.uk/

F Chen, CA Suttle and SM Short. 1996. Genetic diversity in marine algal virus communities as revealed by sequence analysis of DNA polymerase genes. Appl. Environ. Microbiol. 62: 2869-2874

Suttle, C.A., A.M. Chan, and M.T. Cottrell 1991. Use of Ultrafiltration to isolate viruses from seawater which are pathogens of marine phytoplankton. Applied and Environmental Microbiology 57: 721-726.

Wilhelm, S.W. and L. Poorvin 2001. Quantification of algal viruses in marine samples, p. 53-66. *In* [ed.], J. Paul Methods in Microbiology. Academic Press.

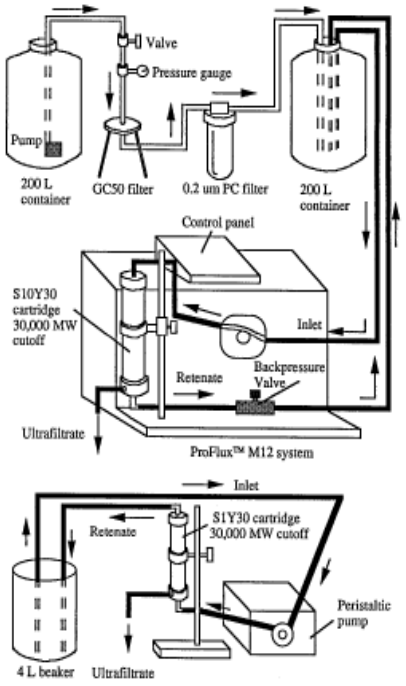


FIG. 1. The ultrafiltration systems used for concentrating natural virus communities from seawater. (Top) Approximately 200 liters of seawater was prefiltered through GC50 glass fiber and 0.2-μm-pore-size polycarbonate (PC) filters, and then the viruses in the filtrate were concentrated into 1 to 2 liters by ultrafiltration with an S10 cartridge mounted on an Amicon ProFlux M12 system. (Bottom) In some cases, the viruses were concentrated into a smaller volume (100 to 200 ml) by a second-step ultrafiltration using an S1 cartridge and a peristaltic pump. MW, molecular weight.

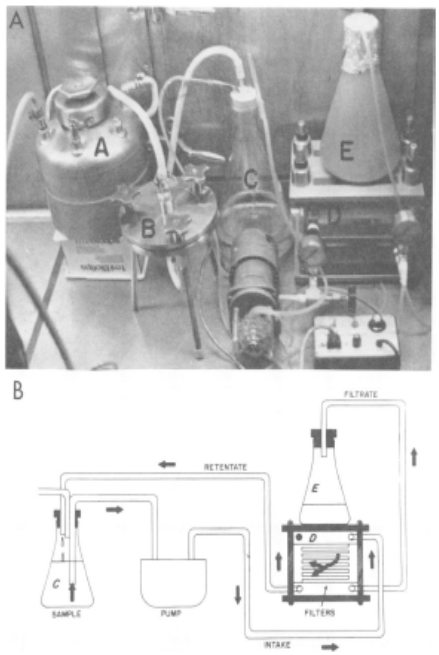


FIG. 1. *(A) Concentration apparatus assembled in a P-4 containment hood line. A, Crude-sample holding tank; B, tripod filter holder containing two Millipore prefilter pads; C, clarified-fluid holding tank; D, Millipore Pellicon Cassette Molecular Filtration unit and peristaltic pump with rate controller (not labeled); E, filtrate holding tank. (B) Diagrammatic sketch of molecular filtration unit.*

Session 1: Sampling equipment demonstration (mesocosm)

Demonstrator: Willie Wilson

Purpose: A brief talk through of sampling equipment used during the course of any fieldwork exercise.

We will collect a range of sampling devices from around the lab to give you an idea of the type of equipment required for sampling. I have been on numerous fieldwork campaigns and it is always interesting to see some of the ingenious devices that are usually hand-made for specific purposes. Most people are probably familiar with the most standard sampling device for oceanographic fieldwork, the CTD recorder: which stands for Conductivity-Temperature-Depth recorder.

FIGURE Lowering a CTD from the side of a ship.
In recent years, CTD instruments have become integral in measuring water salinity, temperature, pressure, depth and density. As the CTD instrument is lowered through the water (or as it sits still at a given point), measurements of conductivity, temperature and depth are recorded continuously. The core CTD instrument is usually surrounded by up to 24 Niskin bottles that can be triggered at defined depths in the water column, each bottle collecting up to 20 litres of water.

One recent Plymouth Marine Laboratory cruise focused on the very surface of the ocean ie. the top two metres plus the surface 'microlayer'. A range of specialised sampling equipment was required for this cruise and will be discussed briefly during the session:

Garrett screen
The Garrett screen sampler is a handheld device consisting of a 50cm square frame enclosing a stainless steel mesh (see figure below).

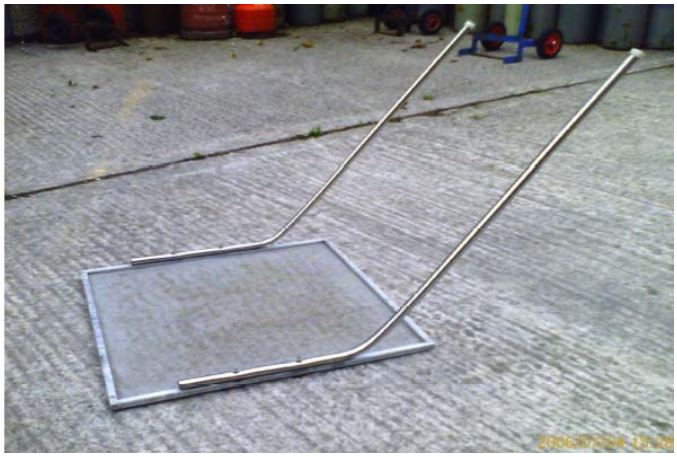


FIGURE: Garrett screen sampler

The screen is slid gently through the water surface at an angle, and then lifted flat back through the surface. Small rectangular cells of water from the sea surface are captured in the interstitial spaces of a wire mesh by means of surface tension forces. The physical thickness of the microlayer sample collected by the screen is calculated from the void area of the screen and the volume of seawater collected. In our experience, this was typically 300-400 µm. This thickness is determined primarily by the diameter of the screen mesh filaments.

Near-surface sampling device **(NSSD)**
A floating nearsurface sampling device (NSSD) had been constructed at PML (see figure below).

This device consists of a flotation ring (1.2m diameter) supporting a central vertical spar. The spar carries a series of 8 sampling bottles spaced at 20cm intervals (upper 5; lower 3 at c. 30cm), and an array of thermistors spaced at logarithmic intervals between a few mm below the surface and ~2m depth. An analogue of skin temperature (~500µm depth) can be obtained from concurrent remote sensing of sea surface temperature. Above the spar is a control system which continuously logs thermistor data and transmits it up the cable to a host computer on board the ship. The control system also allows for the remote firing of the sampling bottles from the host computer. The NSSD is deployed from the ship and allowed to drift away from the ship on a conductor core tether. The tether carries two conductors for DC power supply to the NSSD and two conductors for RS485 communication between the NSSD and the host computer.

Microlayer Sampling Device **(MLSD)**

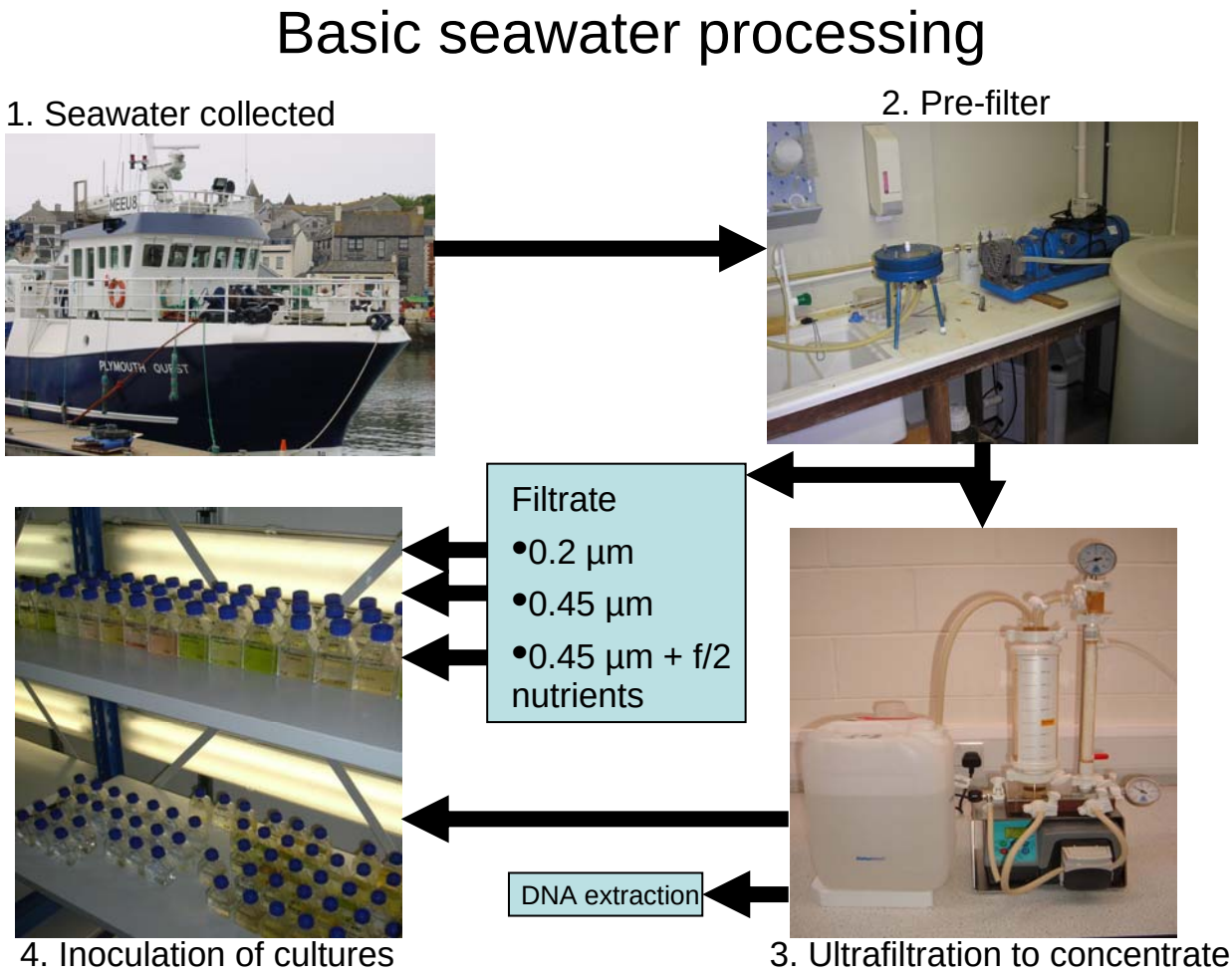
The surface microlayer sampling device (MLSD) is a rotating drum device mounted between the hulls of a small (1.5m long) catamaran platform. The SMS will be deployed tethered to the NSSD. The rotating glass drum picks up water from the microlayer (60-120 μm), which is thenremoved by a Teflon wiper blade and collected in small sample pot. The contents of this pot are continuously removed by a peristaltic pump to a larger (2.7 litre) storage vessel on the upper hull.

Session 2: Protocol for processing a natural seawater sample for virus analysis. (Demonstration)

Venue: Seawater Hall PML

Demonstrators: Steven Wilhelm & Karen Weynberg

Purpose: A brief talk through of processing a water sample from initial collection through to different filtration procedures for inoculation of cultures and nucleic acid extraction. Demonstrators will discuss the pro's and cons of different filter types.



The demonstration will cover the following procedures:

1. **Pre-filtration step.** (in order to remove debris, large zooplankton, etc.).Assemble the stainless steel filter holder and connect to a peristaltic pump. Using ethanol-cleaned blunt-ended tweezers, transfer a 142mm diameter 1.6µm pore size filter onto the support mesh of the stainless steel filter holder.
2. Secure the lid of the filter holder and open the valve on the lid.
3. Start the pump. Close the valve once the filter is primed (water will leak from valve at this point).

4. Collect the filtrate in a clean carboy.
5. **Filtration step**
   This step requires reusable, autoclavable bottle top filter holders for use with 47mm diameter filter membranes. Securely screw the sterile bottle top filter holder onto a 1L glass bottle. Ensure all O-rings of the filter holder are in place.
6. Connect the filter holder apparatus to a vacuum pump.
7. Using sterile tweezers, transfer a 0.45 µm pore size membrane filter to the filter holder. Screw on the upper chamber of the filter unit.
8. To prime the filter and bottle, pour 50ml distilled water into the upper chamber and start the pump.
9. Swirl and discard the filtrate in the bottle
10. Using a measuring cylinder, pour 2x 500ml of the pre-filtered seawater into the upper chamber of the filter holder and screw on the lid.
11. Start the vacuum pump.
12. Stop the pump as soon as 1 litre of water has passed through the filter membrane.
13. Unscrew the upper chamber. Using tweezers remove the filter membrane.
14. Fold the filter membrane and insert into a cryovial.
15. Snap freeze the cryovial in liquid nitrogen. Store at -80°C.
16. Label and keep 1litre filtrate in fridge until ready
17. Repeat filter process for 0.2 µm pore size membrane filter.

1. After use, clean carboys and filter holder units. To clean the carboy, fill with ~250ml 10% HCl, shake vigorously. Leave overnight. Rinse at least 2 times with distilled water.
2. To clean filter rigs, soak overnight in tub of warm ~2% decon or a 5% acid bath. Double rinse with distilled water. (Bottles are cleaned as normal glassware)

Table 3 (opposite) will be used as a starting point for discussions on filter types.

TABLE 3. Infectivity of viruses after filtration through membranes of various types and rated pore sizes on *M. pusilla* and the *Navicula* sp.

Filter[a]	RPS (µm)[b]	Membrane type[c]	Host[d]	
			PD	*Mp*
Gelman Acrodisc	0.2	Polysulfone	±	+
Gelman Supor	0.2	Modified polysulfone	−	−
Gelman Supor	0.45	Modified polysulfone	−	ND
Millipore GVWP	0.22	PVD	−	+
Millipore HVLP	0.45	PVD	+	ND
Gelman GN-6	0.45	Mixed cellulose	−	+
Millipore GSWP	0.22	Mixed cellulose	±	ND
Millipore HAWP	0.45	Mixed cellulose	±	ND
MFS A045A025A	0.45	Cellulose nitrate	−	+
MSI Acetate Plus	0.22	Cellulose acetate	−	ND
Poretics PCTE	0.2	Polycarbonate	+	+
Poretics PCTE	0.2	Polycarbonate (high porosity)	+	ND
MFS GF75	0.7	Glass fiber	+	+
Gelman AE	1.0	Glass fiber	+	+
MFS GC50	1.2	Glass fiber	+	+
MFS GB100R	2.0	Glass fiber	+	+

[a] MFS, Micro Filtration Systems; MSI, Micro Separations, Inc.
[b] RPS, Rated pore size.
[c] PVD, Polyvinylidine difluoride.
[d] The infectivity after filtration is indicated as follows: +, infective; ±, variable; −, no longer infective. ND, Not determined; PD, the *Navicula* sp.; *Mp*, *M. pusilla*.

From Suttle et al. 1991

Session 3. Hands on session for Filtering.

Venue: Room 111 PML

Demonstrators: Susan Kimmance & Ellie Harrison

Purpose: Time to get your hands dirty with a practical session on filtering. Samples collected here will be used for flow cytometry analysis and nucleic acid extraction later in the week.

- Collect 1 L of seawater
- Retain 1 ml of the seawater for the flow cytometry session on Tuesday (see below)
- Link the filter rig to the vacuum pump with tubing
- Fit the filter rig to a clean 1 L collection bottle
- Using blunt ended tweezers set up a 0.45 µm filter on the filter rig
- Filter 1 L of the seawater through the 0.45 µm filter
- Retain 1 ml of the 0.45 µm filtrate for flow cytometry
  Remove 0.45 µm filter from the rig using tweezers
- Fold filter and insert into cryovial for the Denaturing Gradient Gel Electrophoresis (DGGE) session on Friday
- Label cryovial with the date and filter size
- Snap freeze cryovial in liquid nitrogen

- Wash filter rig with Milli-Q
- Fit the filter rig to a clean 1 L collection bottle
- Using blunt ended tweezers set up a 0.2 µm filter on the filter rig
- Filter 1 L of the seawater through the 0.2 µm filter
- Retain 1 ml of the 0.2 µm filtrate for flow cytometry
- Retain filtrate in the 1 L bottle for the Tangential Flow Filtration (TFF) session on Monday
- Label bottle with date and 0.2 µm filtrate
- Store filtrate at 4°C
- Remove 0.2 µm filter from the rig using tweezers
- Fold filter and insert into cryovial for DGGE
- Label cryovial with the date and filter size
- Snap freeze cryovial in liquid nitrogen

- Samples for flow cytometry will be handled as follows:
- Transfer 1 ml of unfiltered water to a cryovial
- Fix 1 ml sample with glutaraldehyde to a final concentration of 0.5 % in a fume hood
- Label vial clearly with date and treatment (either: unfiltered, 0.45 µm or 0.2 µm)
- store at 4°C for 30 min
- snap freeze in liquid nitrogen

Monday 24th July

Time	Activity			Location
0900 - 1000	Case study: Curtis Suttle **VIRUSES INFECTING *HETEROSIGMA AKASHIWO***			Resource centre
1000 – 1030	Methods briefings			Resource centre
1030 - 1100	COFFEE			Mess room
1100	Students split into 3 groups (Red, Green & Blue) **TFF:** Tangential flow filtration (GE Healthcare) **VC:** Virus concentration for TEM (Willie Wilson & Matt Hall) **AFC:** Analytical Flow Cytometry (Corina Brussaard & Claire Evans)			All at the MBA
	TFF Room 5	**VC** Room 82	**AFC** Resource Centre	
1100 – 1230	Red	Green	Blue	
1230 - 1330	LUNCH			Mess room
1500 - 1530	Green	Blue	Red	
1530 - 1600	COFFEE			Mess room
1530-1700	Blue	Red	Green	
1730 - 1900	POSTER SESSION (Prize for best poster) Wine and nibbles included			Resource Centre
1900 -	DINNER			Mess room

Notes:

Monday 24th July

Using Cross Flow Filtration

Demonstrators
Samantha Longshaw, Vikki Ponting, Tanya Hayes (GE Healthcare)

Introduction

Cross flow filtration can be used for a large number of different applications, ranging from the harvest of mammalian cells from a fermentation vessel to the concentration of an antibody in solution, or the concentration of virus particles from a variety of sources.

In contrast to single pass or normal flow filtration (Figure 1), cross flow filtration (Figure 2) involves the recirculation of the feed stream across the membrane surface. The "sweeping action" created by the fluid flow across the surface helps keep the membrane clear and reduces the build up of material which can be seen with normal flow filtration.

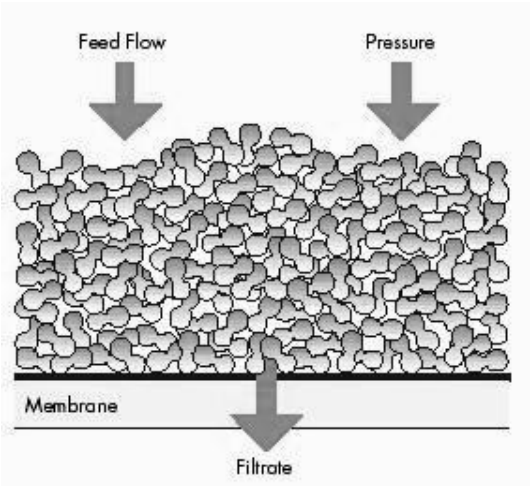


Figure 1: Normal flow filtration. The more material is process the more material builds up on the surface of the membrane, slowing the flow of liquid across the membrane.

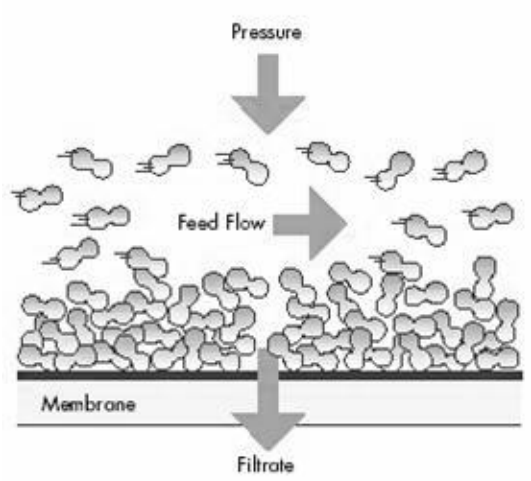


Figure 2: Cross flow filtration. The circulation of the feed stream across the surface of the membrane keeps the membrane surface clear.

The continuous sweeping of the membrane promotes consistent productivity over the entire length of a process.

GE Healthcare manufactures and markets two classes of cross flow membrane separation product. One is based on the hollow fibre cartridge design. The other (the Kvick family) is based on flat sheet membranes. Both ranges comprise devices and systems that can be scaled predictably from volumes of just a few hundred ml to many thousands of litres.

Hollow fibre cartridges and systems are available for both cross flow ultrafiltration and microfiltration applications. They have an open flow path design which can be used to gently process cell suspensions and other particulate feed streams such as viruses.

Kvick (flat sheet) ultrafiltration cassettes enable the user to separate, concentrate and diafiltrate soluble biological solutions.

For applications where the feed stream is either viscous in nature, contains particulate material or is sensitive to shear forces, hollow fibre cartridges are the device of choice. However, if the material to be concentrated or diafiltrated is a soluble protein solution then quicker processing times will be achieved using a flat sheet Kvick cassette.

GLOSSARY OF TERMS

Cross flow
Sweeping action creates fluid flow across the membrane (also called tangential flow).

Feed Stream
Bulk solution to be processed via ultrafiltration or diafiltration (also called process solution).

Retentate
Solution containing species (i.e. virus) retained by the membrane (also called concentrate or reject).

Permeate
Solution containing solvent and solutes which can pass through the membrane (also called filtrate or ultrafiltrate).

Each hollow fibre filtration cartridge consists of a bundle of polysulfone fibres or tubules held in parallel within a plastic housing. The ends of this bundle of fibres are embedded within a resin which allows the free stream to pass through the lumen of the fibres only (Figure 3).

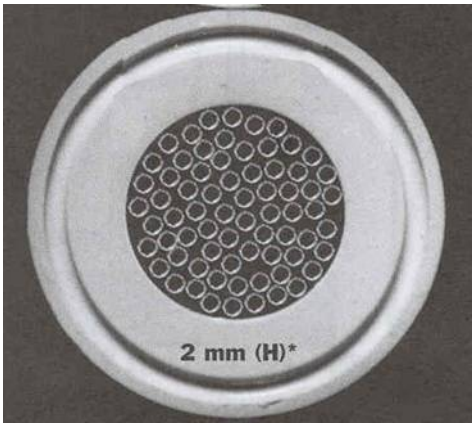


Figure 3: End view of a hollow fibre filtration cartridge.

As the feed stream is pumped through the cartridge, the retentate including any species prevented from passing through the pores of the fibre, continues though a recirculation loop to be passed through the cartridge again. Meanwhile, permeate including any solute which is small enough to pass through the membrane pores, moves from the lumen of the fibre to the shell side of the cartridge (Figure 4).

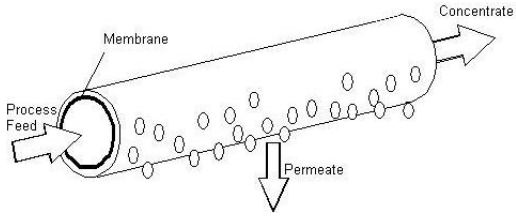


Figure 4: Schematic of individual membrane lumen during operation.

A basic manual system for running ultrafiltration or microfiltration applications consists of a pump, feed

reservoir, permeate collection vessel, pressure gauges and an adjustable valve which allows back pressure to be applied to the system (Figure 5).

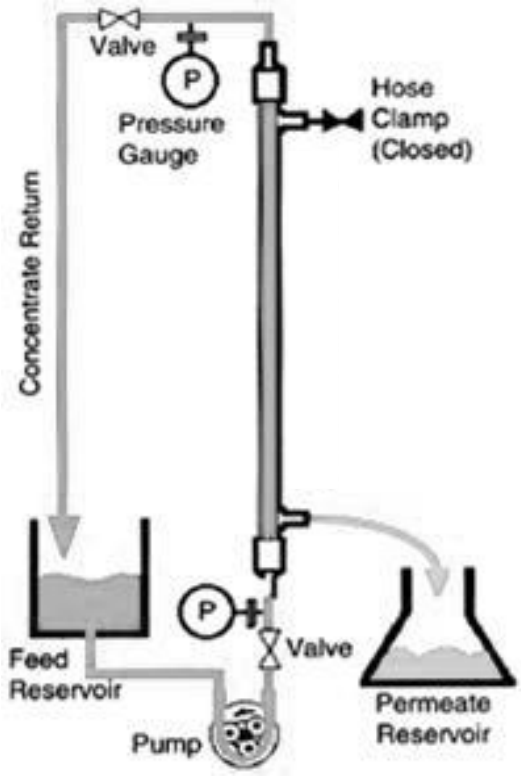


Figure 5: Schematic of manual hollow fibre filtration system.

Monitoring the pressure within the system is important as pressure is used to drive the permeate across the membrane. Pressure is generated from the force of the liquid being pumped into the cartridge and also in certain circumstances by the constriction of the retentate outflow from the cartridge.

The rate at which material crosses the membrane is known as the flux rate. As a convention the flux is recorded in terms of litres of permeate to cross a square meter of membrane in hour (lmh) and can be calculated using the following formula.

$$\text{Flux (lmh)} = \frac{\text{Permeate Flow (ml/min)}}{\text{Cartridge Area (m}^2\text{)}} \times 0.06$$

The flux rate is related to the transmembrane pressure (TMP). TMP is the average of the pressure at the inlet and the outlet of the cartridge minus any pressure applied to the permeate.

$$\text{Transmembrane Pressure} = [(P_{inlet} + P_{outlet})/2] - P_{permeate}$$

When running a hollow fiber cartridge with only pure water, the passage of water across the membrane or water flux will increase linearly with increasing TMP. When processing a feed steam that contain particulates or soluble material the process flux will typically increase as a function of TMP, but only to a point. There will come a point when increasing the TMP will no longer give an increase in flux. This may be a symptom of gel layer formation against the surface of the membrane, restricting the flow of material across the membrane.

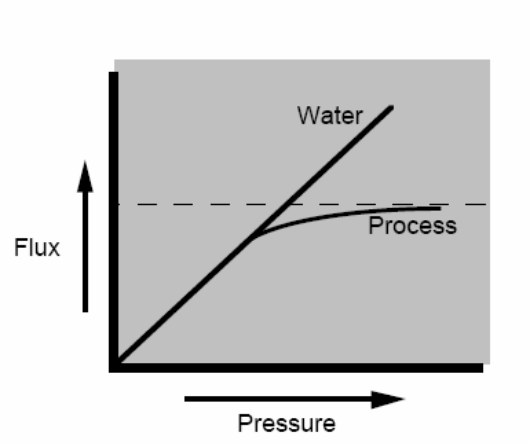


Figure 6: TMP v's Flux rate

Increasing the recirculation rate of the feed stream across the membrane can reduce gel layer formation as can restricting the flow across the membrane by using permeate flow control. You can control the flow though the permeate either by applying backpressure to the permeate or by fitting a pump to the permeate line to take permeate from the cartridge at a defined rate. This can result in a more stable flux rate over the course of your process (Figure 8).

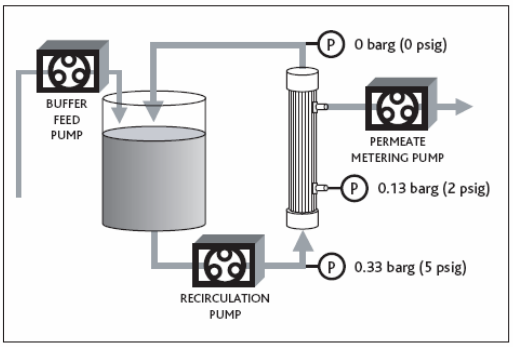


Figure 7: Manual system for permeate flow control.

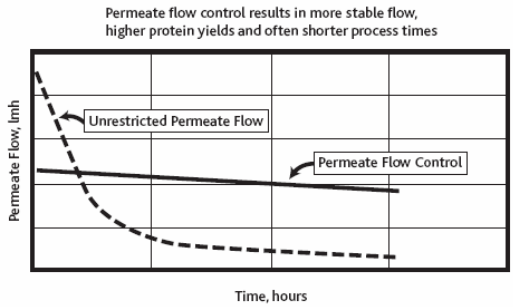


Figure 8: Effect of permeate flow control on process flux rates.

GE Healthcare hollow fiber cartridges are available in a broad spectrum of pore sizes for both UF and MF applications (Table 1). Cartridges come in a range of lengths from 30-120cm, the lumen of the fibers ranges in diameter from 0.25mm to 3.0mm. In all there are over 700 different GE Healthcare hollow fiber cartridges to choose from.

Ultrafiltration (NMWC)	Microfiltration (microns)
1,000	0.1
3,000	0.2
5,000	0.45
10,000	0.65
30,000	
50,000	
100,000	
300,000	
500,000	
750,000	

Table 1: Hollow fiber pore size availability

When choosing the pore size you require it is important to consider whether you require the membrane to retain your molecule/particle of interest or if you want that molecule to pass across the membrane.

If you wish to retain a molecule you should choose a membrane pore size which is 3-5X smaller than your molecule's molecular weight. If you wish your molecule to pass through the membrane you should choose a pore size which is just big enough to let your molecule of interest through, this is usually 5-10 X larger. Choosing too big a pore size will give a high initial flux rate, but less long term flux stability (Figure 9).

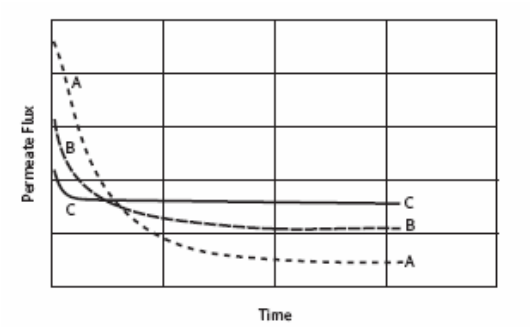


Figure 9: Flux in relation to pore size. Membrane A pore size> membrane B pore size>membrane C pore size.

With particulate or viscous feed streams, lumen diameters of 0.75 to 1.0mm are most suitable. However if the feed stream is very dilute a smaller lumen diameter can be used. Using a smaller lumen diameter can allow you to generate high level of shear at lower flow rates.

Hollow fibre cartridge systems

GE Healthcare provides a range of manual hollow fibre cartridge-based systems, from small systems for laboratory-scale work to larger-scale systems that are modular in design and suitable for GMP production.

MidJet™ Systems are compact and self-contained. They use MidGee™ Cross Flow Filters to facilitate rapid processing of volumes up to 200 ml. Low hold-up volumes allow concentration of volumes as small as 2-5ml.


Figure 10: MidJet Filtration system.

QuixStand™ systems are compact, self-contained units designed to work with Xampler™ hollow fibre cartridges and enable rapid processing of volumes up to 10 litres.


Figure 11: QuixStand Filtration System

FlexStand™ systems accommodate Pilot/Process Scale Cartridges from 0.14 m^2 to 3.4 m^2 for processing volumes from 5 to 100 litres and more.

GrandStand Pilot/Process Systems are self-contained and designed for MaxCell Large Process Scale Cartridges up to 13 m^2. Process volumes range from 50 to 1000 litres and higher.

Hollow fibre cartridges

Since the early 1980s, we have developed more than 20 different cartridge designs: 10 ultrafiltration ratings, four

microfiltration ratings, and six hollow fibre lumen diameters.

MidGee™ and MidGee Hoop Cross-flow Filters give rapid concentration and/or diafiltration of biological solutions up to a volume of 200 ml.

Figure 12: MidGee Hollow fibre cartridge.

With membrane areas from 20 cm^2 to 1400 cm^2, **Xampler™** laboratory cartridges enable rapid concentration and/or diafiltration of biological solutions with a starting volume of less than 1 up to 10 litres.

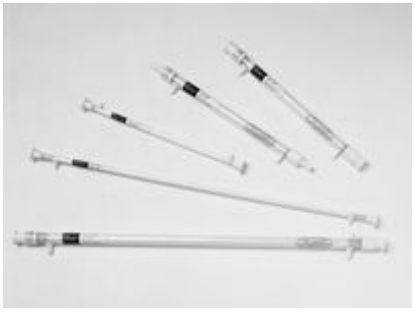


Figure 13: Xampler Hollow fibre filtration cartridge.

MaxCell™ Large Process Scale Cartridges provide up to 13 m^2 of hollow fibre membrane in a single, lightweight housing. They are specifically designed for large scale biomanufacturing operations, and multiple cartridges can be arranged in parallel to ensure uniform separations performance.

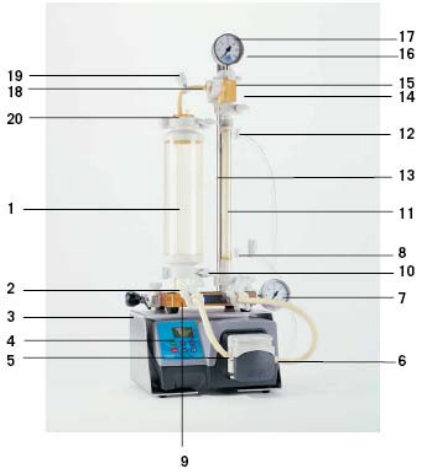
ProCell™ large process Scale cartridges are 15cm diameter cartridges for large production scale ultrafiltration and microfiltration applications.

QuixStand Demo

The instrument we are going to use today is the QuixStand. This is being used in combination with a Xampler cartridge to concentrate viral particles from sea water samples.

System Overview (typical)

Figure 14: Typical QuixStand Configuration



1 Feed reservoir (1000 ml shown)
2 Permanent reservoir attachment
3 Pump cover
4 Pump controls
5 Pump inlet
6 Pump outlet
7 Inlet pressure gauge
8 Permeate outlet
9 Lower manifold
10 Sanitary connection
11 Xampler cartridge (Size 4M shown)
12 Permeate outlet
13 Support rod
14 Tightening knob (not visible)
15 Upper manifold assembly
16 Support rod cap (not visible)
17 Outlet pressure gauge
18 Retentate outlet
19 Backpressure valve
20 Reservoir cap, gasket and clamp

System assembly

The system should be assembled as detailed in the QuixStand Manual (18-1174-68). Once assembled the system may be autoclaved (autoclavable QuixStand Only) or sanitised using sodium hydroxide.

Initial Start Up

1. Close sampling/drain valve, secure cartridge in upper and lower manifolds. Ensure the pump tubing is correctly positioned and tensioned within the pump head.
2. Confirm flexible tubing is connected from the retentate outlet on the upper manifold to one of the barbs on the reservoir cap. If the process solution tends to foam, ensure there is a retentate downcomer pressed into the retentate cap for the retentate line.
3. Direct the flexible tubing from the upper permeate line to a collection flask. If you chose not to use the lower permeate line, close it off with one of the covers provided.
4. Remove the sanitary clamp from the reservoir cap and slide it to one side.
5. Add the feed solution to the reservoir.
6. Reposition the reservoir cap on the silicone gasket and clamp in place using the sanitary clamp.
7. Make sure the back pressure valve on the retentate line is completely open (turn counter clockwise).
8. Start the pump on slow speed and wait about 30 seconds for the pressure to build up. The pressure gauges are mechanically dampened and respond slowly. Gradually increase the pump speed. The inlet pressure will build, while the outlet pressure gauge may still read zero.
9. Apply back pressure by slowly closing the back pressure tubing valve (additional back pressure is not required in all cases). This will cause the pressure at the inlet and the outlet to rise.
10. Adjust the feed flow rate and back pressure to achieve the desired process conditions. Monitor the inlet pressure gauge. If the pressure rises too high, lower the pump speed.

Table2: Xampler cartridge specifications.

Housing Size	Lumen Diameter (mm)	Membrane Area (cm^2)	Membrane Area (ft^2)	Hold-up Volume Lumen (ml)	Hold-up Volume Shell (ml)	Permeate Port (inches)
3M	0.25	370	0.40	3	7	¼
	0.5	140	0.15	2	9	¼
	0.75	120	0.13	3	8	¼
	1	110	0.12	3	5	¼
3X2M	0.5	290	0.31	5	14	¼
	0.75	260	0.28	6	14	¼
	1	225	0.24	6	12	¼
4, 4M	0.25	1200	1.29	10	42	⅜••
	0.5	650	0.7	8	45	⅜••
	0.75	460	0.5	10	40	⅜••
	1	420	0.45	12	30	⅜••
4X2, 4X2M	0.5	1400	1.5	20	75	⅜••
	1	850	0.9	30	50	⅜

Table 3: Xampler cartridge nominal feed flow rates.

Housing Size	Lumen Diameter (mm)	Shear Sensitive (ml/min)	Low Fouling (ml/min)	High Fouling (ml/min)
3M, 3X2M	0.25	50	110	230
	0.5	60	120	250
	0.75	100	200	400
	1	150	300	600
4, 4M, 4X2, 4X2M	0.25	190	380	760
	0.5	300	600	1200
	0.75	400	800	1500
	1	600	1200	2500

[1] Shear rates for shear sensitive = 2000 sec^{-1}; low fouling = 4000 sec^{-1}; high fouling = 8000 sec^{-1}. Flow rate/pressure values determined with water will vary from experimental solutions due to changes in temperature, viscosity and degree of suspended solids.

The feed flow rate you use will be dependent on your sample type; Table 3 gives details of the recommended feed flow rates for different sample types. If your sample is sensitive to shear (which many virus samples are) then you should use a low feed flow rate. If your sample is viscous then a high feed flow rate should be used to avoid membrane fouling.

Constant Volume Filtration/Diafiltration

If your sample volume is too large to fit into the reservoir all at once, then you can configure the QuixStand system to draw extra sample into the reservoir as material leaves through the permeate. This method can also be used for constant volume diafiltration.

In order to configure the QuixStand for this follow these steps:

1. Attach tubing from your diafiltrate solution/extra sample to the empty hose barb connector on the reservoir cap.
2. Turn on the pump to start processing. As the process continues extra sample or diafiltrate buffer will be drawn into the reservoir. Care must be taken to ensure that the lid of the reservoir is clamped securely in place.
3. If the diafiltration solution causes foaming in the feed reservoir, ensure the second downcomer is attached to the bottom of the reservoir cap. Ensure the downcomer extends down below the fluid level already in the reservoir.

Process conditions for Marine Viruses

Work carried out to recover viruses from sea water has found that the virus particles are sensitive to shear and prone to poor recovery from the membrane. This makes hollow fibre cartridges ideal for this application as they work at low shear rates compared to flat sheet devices. Hollow fibre cassettes also lack internal screens which can cause problems in the recovery of particulate samples.

The conditions used in this demonstration were as follows:

Cartridge –
……..

Feed flow rate -
……..

Back pressure –
……

Inlet pressure (PO) -
……..

Outlet pressure (PI) –
……..

Transmembrane Pressure (TMP)
……..

Permeate flow rate –
……..

Flux rate -
……

Demonstrators

Samantha Longshaw,

Vikki Ponting, Tanya Hayes

Monday 24th July

Concentration of viruses onto electron microscope grids.

Location: MBA 73, 82 and 83

Demonstrators: Willie Wilson / Matt Hall

Purpose: To concentrate viruses onto electron microscope grids for morphological analysis in the electron microscope.

Introduction:

There are several steps involved in the characterisation of any virus-host interaction and subsequent downstream ecological effects. One crucial initial step is to determine if viruses are indeed present in a targeted environmental system and can be identified morphologically by visual inspection using transmission electron microscopy (TEM). At this stage putative viruses are termed virus-like-particles (VLPs). Bergh et al. (1989) is perhaps one of the most cited papers that demonstrates the high abundance of viruses in aquatic systems. This celebrated paper, from the laboratory of Professor Gunnar Bratbak used electron microscopy to illustrate high concentrations of viruses. But it is not as easy as just putting a drop of water onto an electron microscope grid. Gunnar's team pioneered the use of ultracentrifugation to concentrate viruses on a grid prior to analysis (the method is described in more detail in their later paper: Borsheim et al, 1990).

The secret was to make a solid platform at the base of the centrifuge tube using an epoxy resin, once that had solidified you could fill it with the water of your choice, sink some grids into it then centrifuge at high speed. Viruses, bacteria, phytoplankton in the water sample were all spun onto the grid and using a good electron microscope you can see an incredible diversity of viruses and other microorganisms. In the early days of virus ecology (1990s!) this tool was used to count viruses and bacteria (eg. Bergh et al 1989; Wilson et al. 2000) though I don't know of anyone who does this now, particularly with the advent of epifluorescence microscopy and flow cytometry (both will be taught on this course) as such accurate tools for virus enumeration. TEM will always be an invaluable tool to gain morphological information about viruses and possible most importantly, generate nice images! (eg. The front cover of the special issue of JMBA on aquatic viruses contains an image using this method – see following page)

Concentration of viruses onto electron microscope grids is an incredibly simple tool and will be demonstrated to you in this session. We hope to use the grids made today to look at viruses from the English Channel in the electron microscopy session tomorrow.

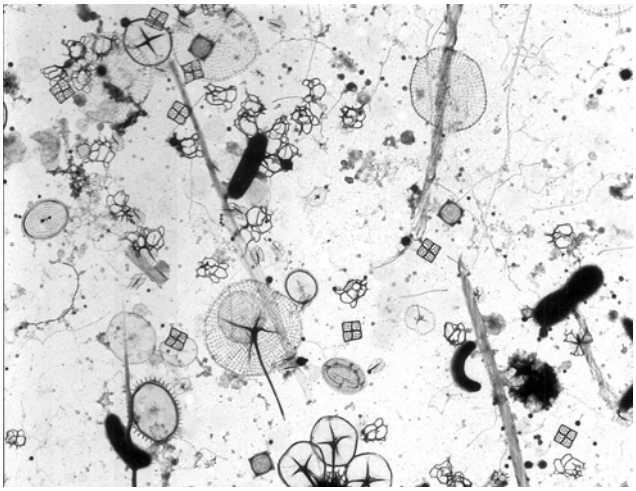


Figure: Cover image of the JMBA special issue on Aquatic Viruses (you have a copy in your registration packs). You need to look beyond the bacteria and elaborate phytoplankton scales to reveal the abundance and morphological diversity of viruses from this Norwegian Fjordic seawater sample. It was electron microscope images like this that inspired the recent explosion of research in aquatic viruses. At first glance the image reveals box-, crown- and limuloid scales from *Pyramimonas orientalis*, box scales from *Pyramimonas cirolanae*, and different body scales from *Heterocapsa* sp. and *Chrysocromulina* sp. However, a closer look at the image will reveal bacteria and a wide morphological range of virus-like particles; by far the most numerous component of any seawater sample. The particles were harvested by ultracentrifugation directly onto 400 mesh Ni grids supported with carbon coated formvar film, positively stained with uranyl acetate and air dried (courtesy of Gunnar Bratbak and Mikal Heldal, University of Bergen).

Method at a glance (from Wilson et al 2000)

Bacteria and VLPs in preserved samples were examined by TEM following ultracentrifugation of the particles directly onto formvar-coated copper grids (Borsheim et al. 1990). Centrifuge tubes were modified by moulding a flat supporting bottom of two-component epoxy glue (slow setting Araldite®) by centrifuging the glue onto the bottom of the tube then leaving to set. Modified centrifuge tubes were filled with preserved water and formvar-coated grids attached to nitro-cellulose filter paper were submersed below the surface and left to sink with the copper grid upwards. The samples were centrifuged in a Beckman L8-70M ultracentrifuge using a swing-out SW40 rotor at 30,000 rpm (160,000 x g) for 1h at 10°C. Following centrifugation, the supernatant was poured off and the grid was air dried prior to staining with a saturated solution (approx. 3% w/v) of uranyl acetate. TEM analysis was conducted on a Jeol JEM 200CX TEM operated at 160kV at magnifications ranging from x20,000 to x100,000. For counting of VLPs, view fields were randomly selected at a magnification of x37,000 and counted until the total counts exceeded 400. Bacteria were counted concurrently in the same fields of view to calculate VLP:bacteria ratios in each of the samples.

Materials required:
Two-component epoxy resin (we use slow setting Araldite® precision 2011)
Lollipop sticks
Polyallomer Centrifuge tubes (Beckman Instruments 331374)
Benchtop Centrifuge
Balance
Beaker
Double-sided sticky tape.
Scalpel blade.
13 mm diameter nitrocellulose filters (Whatman 7184-001)
Petri-dishes
200 mesh Formvar-coated Copper grids. (Agar Scientific S162)
Grid tweezers.
3mm filter paper
Water sample to look at (15 ml).
Ultracentrifuge
Swing-out rotor (eg SW40)
Long spatula
Grid box

Method:
Step 1: Make the platform
- Mix a resin in equal amounts, approximately 1ml required per tube.
- Spoon inside the centrifuge tube (wipe off any from the outside of the tube or it will stick to the rotor!) use scales to make sure the tubes are balanced (place tubes into a beaker to help the stand up).
- Centrifuge in a swing out rotor for 10 minutes at 6,000 rpm. Leave to set either in a drying oven or at room temperature (make sure the tube is level – a good rack or stick to a piece of blue tak)
- Once set that's it ready for the next step (this takes a day, so you won't be using these ones today).

Step 2: Prepare grids:
- Place a thin sliver of double-sided sticky tape in the centre of a 13mm diameter filter.
- Carefully stick the very edge of a formvar-coated nickel grid to the tape so that it just touches the tape (otherwise you will tear off the formvar from the grid).
- [Usually I would also stick a second grid on the tape – but no need to do that today].
- Cut a circle of 3mm paper so it fits neatly into a petri dish. Soak thoroughly with MQ water.
- Place the filter on the wet 3mm paper to soak it (this step is necessary to allow the filter to sink in the next step)

Step 3: Sink the grids:
- This is the fun bit and takes years of practise to get right!
- Fill your platform-containing centrifuge tube with a seawater sample (to within 5 mm of the top).

- Tap on the bench to get rid of any bubbles (important!)
- Place your filter in the tube at an angle (45°?) and tap gently so it sinks down to the platform. The trick is to make sure it doesn't flip over, if anyone finds a sure-fire way of doing it please let me know!!!

Step 4: Centrifugation (there will probably be ***no*** time to do this in the 90-minute session):

- Balance the tubes (very important).
- Centrifuge at 30,000 rpm (approx. 160,000 x g) for 1h at 10°C in a swing-out SW40 rotor.
- Once centrifugation is complete, pour off the supernatant and leave tubes upside-down to remove remaining liquid.
- Use a spatula to remove the filter.
- Carefully pick off the grid and place in grid box ready for staining prior to TEM.

Useful references:

Bergh O, Borsheim KY, Bratbak G, Heldal M (1989) High abundance of viruses found in aquatic environments. Nature 340: 467-468

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Wilson, W.H., Lane, D., Pearce D. and Ellis-Evans, JC (2000) Transmission electron microscope analysis of viruses in the freshwater lakes of Signy Island, Antarctica. Polar Biology 23: 657-660.

Analytical flow cytometry

Corina Brusaard, Claire Evans

Bacteriophages: Methods and Protocols

Editors: M. Clokie and A. Kropinski

part of the series Methods in Molecular Biology, published by Humana Press, USA

Chapter B-10:
Enumeration of bacteriophages using flow cytometry

Corina P. D. Brussaard

Abstract

Rapid identification and enumeration of the numerically important bacteriophages has been till recently a major limitation for studies of virus ecology. The development of sensitive nucleic acid stains, in combination with flow cytometric techniques, has changed this. The flow cytometric method allows the detection and discrimination of a wide variety of viruses of different morphology, genome type and size. The present paper describes an optimized protocol for the enumeration of bacteriophages using a standard bench-top flow cytometer.

Key Words: Bacteriophage, Enumeration, Detection, Flow cytometry, Green fluorescence, Methodology, Nucleic acid-specific staining, Protocol, Viruses

1. Introduction

The study of viruses that are relevant pathogens for humans, animals or plants has received much attention for a long time already. Only recently, it became clear that viruses are very abundant in aquatic environments (10^5 - 10^8 ml^{-1}) and highly dynamic in both total numerical abundance and diversity (1-5). Viruses appear important regulating components in population dynamics, diversity, succession, gene transfer and geochemical cycling of elements (2-4, 6-8). An assay allowing rapid enumeration of virus particles is logically most beneficial for studies of viral ecology. The more traditional methods for virus quantitation include transmission electron microscopy (9-11), the use of antibodies, plaque counts, and most-probable-number assays. These techniques are, however, typically very time-consuming. And although the latter three assays have the advantage of detecting infectious virus particles, they are host-specific and/or culture-based. The introduction of high fluorescence-yield nucleic-acid-specific stains has allowed a more rapid identification and enumeration of the total virus community using epifluorescence microscopy (11, 12). Recently, the development of an assay combining the use of these sensitive nucleic acid stains

with flow cytometry (13-16) has resulted in rapid speed analysis with high precision and reproducibility. Direct comparison with epifluorescence microscopy and electron microscopy showed that total virus counts were highly comparable (17).

The flow cytometric assay allows the discrimination of various virus groups in natural samples based on their green fluorescence (16). But the method is also widely applicable as clearly shown by Brussaard et al. (14). A large variety of viruses of different morphologies, genome type and size could be detected by flow cytometry.

The protocol presented here is based on earlier studies (14, 16), executed on different viruses (including many bacteriophages) in order to provide an optimized and consistent method. That study clearly showed that optimal detection of virus particles depended on more than one factor, including type and concentration of fixative and dye, method of storage, type of solution used to dilute the sample, incubation temperature and duration. Although recommended to specifically determine which conditions are optimal when analysing specific phage species, one set of variables provided the best results for mixed bacteriophage samples. In summary, samples should be fixed with glutaraldehyde (0.5% final concentration, 15-30 min at 4°C), frozen in liquid nitrogen, stored below -80°C, at least 10-fold diluted in TE buffer (pH 8), stained with SYBR Green I at a final dilution of 5×10^{-5} the commercial stock, incubated for 10 min in the dark at 80°C, and cooled for 5 min prior to analysis.

2. Materials

Preservation of samples and storage

1. Adjustable pipettes + tips: 100-1000 µl for sample, 2-20 µl for fixative.
2. Glutaraldehyde 25%, EM-grade (Merck). Aliquot (to prevent contamination) and stored at 4°C in fixative fridge. *See* **Note 1** for safety measures.
3. Sterile cryovials, 1-2 ml (Greiner Bio-One, Inc.).
4. Fridge (4°C), for fixed samples.
5. Liquid nitrogen.
6. Freezer, –80°C.

Working stock solutions

SYBR Green I

1. SYBR® Green I nucleic acid gel stain, 10,000X concentrate in DMSO, commercial stock (Molecular Probes, Inc.). *See* **Note 2** for safety measures.
2. Sterile MilliQ (*see* **Note 3**).
3. Microcentrifuge.
4. Sterile reaction tubes (Eppendorf vials), 1.5 ml.
5. Adjustable pipettes + sterile tips: 100-1000 µl, 2-20 µl.
6. Gloves.
7. Waste container for fluorescent dye solid waste.

Internal reference

1. FluoSpheres carboxylate modified microspheres (beads), 1.0 μm, yellow-green fluorescent (Molecular Probes, Inc.). Stored in the dark at 4°C.
2. Sonicator bath (or Vortex).
3. Sterile MilliQ.
4. Sterile tubes (5 and 15 ml).
5. Adjustable pipettes + tips: 100-1000 μl.

TE-buffer

1. TRIS, 1 M, pH=8.0 (Tris-base, m.w. = 121.10; Boehringer Mannheim).
2. EDTA, 0.5 M, pH=8.0 (m.w. = 372.24; Sigma-Aldrich).
3. MilliQ.
4. Bottles with lid, 500 ml.
5. Autoclave.
6. Syringe, 50 ml.
7. Sterile membrane filter of 0.2 μm pore-size (Sterile FP30/0.2μm Schleicher & Schnell).
8. Sterile bottle with lid, 50 ml.

Prestart

Getting ready of the flow cytometer (FCM)

1. Flow cytometer (FCM) with 488 nm Argon laser (benchtop FacsCalibur, Becton Dickinson, Inc.). *See* **Note 4** for more information.
2. FCM tubes, 5 ml (Becton Dickinson, Inc.)
3. Tissues.
4. MilliQ.
5. Cleaning solutions for BD FCMs: BD™ FACSClean and BD™ FACSRinse

Calibration of flow rate

1. Becton Dickinson flow cytometer with 488 nm Argon laser.
2. FCM tubes, 5 ml (Becton Dickinson, Inc.).
3. Balance plus beaker glass (to put the sample tube in for weighing).
4. MilliQ.
5. Sterile and filtered TE-buffer 10:1, pH=8.0 (see 2.2.3.).
6. Chronometer.

Enumeration of viruses

1. Flow cytometer with 488 nm Argon laser.
2. FCM tubes, 5 ml (Becton Dickinson, Inc.).
3. Adjustable pipettes + tips: 100-1000 μl, 20-200 μl, 5-50 μl.
4. Tube racks (should be able to stand 80°C).
5. Sterile and filtered TE-buffer 10:1, pH=8.0 (see 2.2.3.).
6. MilliQ.
7. Tissues.
8. Water baths, set at 35^{o}C and at 80^{o}C.

9. Waste containers for fixative- and fluorescent dye-contaminated solid waste (e.g. tips) and liquid waste (samples).
10. Notation forms (*see* **Note 5**).
11. BD™ FACSClean, BD™ FACSRinse and BD™ FACSFlow.

3. Methods

Viruses are too small in particle size to be discriminated solely on the basis of their scatter properties using the standard commercially available bench top flow cytometers. The new generation of fluorescent nucleic acid gel stains are high-sensitivity reagents, emitting intense fluorescence when intercalated with DNA and RNA. This qualifies them for many applications where the amount of nucleic acids is limiting, such as the detection of viruses. Their low background fluorescence, furthermore, adds to their usefulness. Especially the visible excitation maximum of SYBR Green I dye–stained nucleic acids near 497 nm is very close to the principal emission lines of many laser-scanning instruments, such as the Argon-ion laser (488 nm) bench top flow cytometers that are used to detect and enumerate viruses (*see* **Note 6**).
Noteworthy to mention in this respect is the finding that different phage species revealed variable green fluorescence; sometimes comparable despite different genome size, and sometimes different despite comparable genome size (16). The green fluorescence of stained phages is thus not linearly related to genome size. Although customary for stained bacteria, reference to high-DNA viruses and low-DNA viruses should be avoided at all times (better is to refer to high and low (green) fluorescent virus populations).
Because flow cytometers are basically not designed for the analysis of these small and abundant particles, attention to detail must be given in order to obtain data of high quality. An ultrasensitive stain and low background fluorescence (a high signal to noise ratio) are then of primary importance, but the aspect of optimal working conditions and thorough cleaning of the flow cytometer's flow cell should not be overlooked. Working close to the lower detection limit, it is, furthermore, essential to run with settings that do not generate electronic noise. It is, therefore, crucial to determine the level of background noise (Fig. 1). This can be accomplished through enumeration of sterile, 0.2 μm pore-size filtered seawater (of comparable composition) or any other liquid equal to the actual sample. Ideally, one would use 0.02 μm pore-size filtered natural sample for the blank, but often the filtration procedure is difficult and generates substantial background noise.
The addition of a mild surfactant at low concentration occasionally improves the coefficient of variance of the green fluorescent signal and thus may improve discrimination of different virus populations. Because the drawback with the addition of detergents is the generation of background noise, care should be taken using detergents when counting phage samples with relatively low green fluorescence (*see* **Note 7**).
Because reproducibility and accuracy are important when analysing relatively low-fluorecent phage particles, it is essential to work fast in order to keep the time between staining and cooling of the sample, and actual analysis as short as possible.

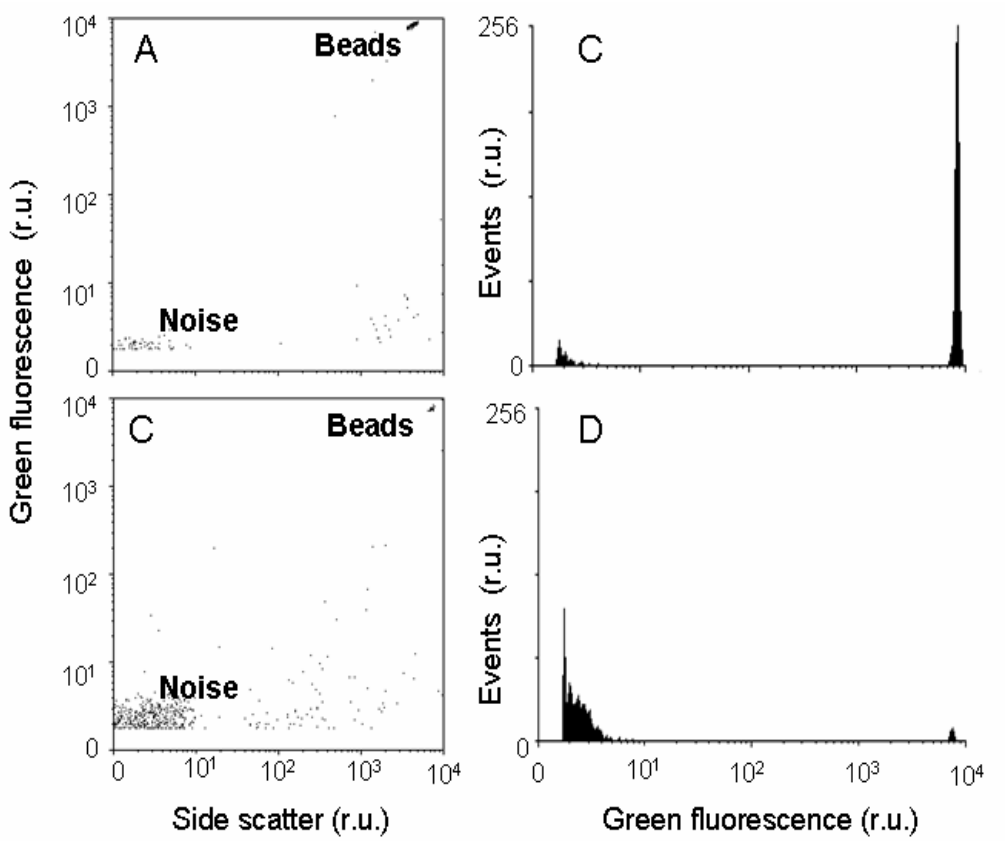

Fig. 1. Biparametric plots of side angle scatter versus green fluorescence. (A) FluoSpheres carboxylate modified 1.0 μm microspheres (Molecular Probes, Inc.) form a tight cluster and are used as internal standard. (B) Blank for natural seawater samples: 0.2 μm TE-buffer (pH 8) with the appropriate volume of sterile 0.2 μm seawater (100-fold diluted), beads as internal standard (10 μL per 1 mL total volume), and SYBR Green I as dye (final concentration 5 x 10^{-5} the commercial stock).

Reproducibility should also be determined (*see* **Note 8**). Standard deviations for total virus counts are generally <5%, determined for a variety of virus samples, including natural samples with mixed phage communities (Brussaard unpubl. data).

Preservation of samples and storage

1. Carefully pipette 0.5-1 ml of sample into cryovial. If possible prepare replicates to have the opportunity to reanalyse if necessary.
2. Add 25% EM-grade glutaraldehyde (*see* **Note 9**) to a final concentration of 0.5% (20 μl to 1 ml sample).
3. Allow fixation for 15-30 min at 4°C.
4. Freeze sample in liquid nitrogen (N_2) and store at –80°C (*see* **Note 10**).

Working stock solutions

SYBR Green I

1. Thaw the commercial stock solution of SYBR Green I in the dark at room temperature, mix by vortexing for 10 sec., followed by a short spin in a microcentrifuge.
2. Prepare a working stock solution by adding 5 µl of the commercial stock to 995 µl sterile MilliQ in sterile Eppendorf vials. Work in dimmed light (SYBR Green I is light sensitive). Prepare several working stock vials at once and store at –20°C till use.

Internal reference

1. Mix the commercial stock of beads rigorously as the beads tend to aggregate: preferably sonicate briefly and otherwise use a Vortex shaker.
2. Prepare a primary stock solution by adding 1-2 drops of the commercial stock into 10 ml sterile MilliQ in a sterile tube (e.g. 15 ml plastic Greiner or Falcon tube). This primary stock can be stored at 4°C for long periods of time, but do check the quality of the beads prior to use by running a small subsample of the working stock diluted in TE-buffer through the FCM at the appropriate settings (see 3.4.). The beads must give a tight population with a specific green fluorescence, as shown in Fig. 1.
3. Prepare for daily use a working stock by adding 10 µl primary stock to 2.5 ml sterile MilliQ or TE-buffer (10:1, pH=8.0) in a 5 ml sterile tube with lid. The working stock can be kept outside the fridge for the entire day. Do mix every time before use.

TE-buffer 10:1 (10 mM Tris, 1 mM EDTA)

1. Prepare 500 ml TE-buffer 10:1 by adding 5 ml of 1 M Tris (pH=8.0) and 1 ml of 0.5 M EDTA (pH=8.0) to 494 ml of MilliQ. Mix well and check pH (should be 8.0).
2. Divide over 2 bottles of 500 ml and autoclave for 20 min with the lids closed. Although it is this way rarely necessary to adjust the pH afterwards, do check the pH before use (adjust if necessary to pH=8.0). Store at room temperature.
3. Filter a small volume of the TE-buffer (50 ml) through a 0.2 µm pore-size filter into a sterile bottle (or tube) prior to use. Check the quality of the solution before use by running a nonheated, as well as a heated subsample of the TE-buffer through the FCM at the appropriate settings (see 3.4.). Both samples should not give more than 30 events sec^{-1} at a flow rate of approximately 35 µl min^{-1}.

Prestart

Getting ready of the flow cytometer

1. Start with checking the sheath and waste containers: empty the waste container and fill the sheath container with freshly prepared MilliQ. Because the samples are fixed, MilliQ can be used as sheath fluid. It contains insignificant numbers of particles and is cheaper than TE (the solution the virus samples are diluted in).

2. Turn on the FCM, pressurize and wipe the outer sleeve of the sample inlet (sampler). Place a tube with MilliQ under the sampler, and let the machine run for at least 10 min.
3. Take away the sample tube, wipe the sampler and replace with a new tube newly filled with MilliQ. Check whether the machine is clean enough to allow the enumeration of viruses by setting the trigger on side scatter (SSC) and the voltage below, but close to, the level where electronic noise starts to become significant (*see* **Note 11**). Typically, the SSC voltage is set around 300; the event rate at a flow rate of 35 µl min^{-1} should then be <75 events sec^{-1}.
4. Only when the FCM is considered clean according to the above criteria is precise enumeration of phages secured. If the event rate is higher, the machine is still too dirty and the machine should be cleaned before use: run 10 min BD™ FACSClean, followed by 10 min BD™ FACSRinse, and 10 min MilliQ. Test again with a new subsample of MilliQ. If the machine is still dirty, try a longer cleaning run, primer several times (after removing sample tube), and/or contact the responsible person for advice about a more rigorous cleaning procedure. Depending on what other type of samples are analysed, the FCM can be rather dirty according to the standards of those interested in enumerating viruses. In this case, be prepared that cleaning may become an important activity prior to the actual use of the machine, which can take quite some time.

Calibration of flow rate

1. Make sure the waste container is empty and sheath fluid container is filled with MilliQ.
2. Select the appropriate flow rate. The typical flow rate is MED, between 25 and 35 µl min^{-1}, which is a good intermediate between speed, statistical number of phages counted, and precision (the lower the flow rate the better the precision).
3. Fill a FCM tube with 2-3 ml of TE and determine its weight (X_0).
4. Remove the outer sleeve of the sampler carefully and wait until a droplet falls. Before the next droplet forms, put the tube under the sampler and place the sample arm in the 'run' position. Simultaneously, start the chronometer.
5. Run the sample for at least 15 min. Remove the tube and stop the chronometer.
6. Weigh the tube and calculate the flow rate (µl min^{-1}) using the formula:

$$\frac{\left(X_i - X_f\right) \times 1000}{t} = flow\,rate$$

where X_o = initial weight, X_1 = final weight, and t = time (min). *See* **Note 12**.

Enumeration of viruses

1. Turn on the 80°C and the 35°C water bath.
2. Allow time for the SYBR Green I working solution to thaw (in the dark at room temperature). Although the SYBR Green I working stock solution can theoretically withstand repeated freezing and thawing, it is recommended to reuse the same working stock vial only once in order to maintain optimal staining quality.
3. Take a set of samples out of the –80°C freezer and thaw them relatively quickly (1-2 min) in water of about 35°C (thawed samples should still be cool). A set of 8 samples at a time works very well (*see* **Note 13**).
4. Prepare a dilution series (0.5-1 ml per tube) for each sample in TE-buffer in order to optimize the staining (*see* **Note 14**) and to prevent coincidence of the phages during analysis (*see* **Note 15**). Make sure to minimize the error due to low-volume pipetting, and dilute at least 10-fold (and preferably >25-fold) when working with seawater samples (*see* **Note 16**). However, very high dilutions not only require longer analysis time, but also result in loss of the emission signal of the nucleic acid-dye complex. Typically, the optimal event rate is between 200-600 events sec^{-1}.
5. Always add a blank of TE-buffer with, according to the dilutions prepared, the appropriate volumes of sterile 0.2 μm filtered reagent equal to the actual sample but without the phages (e.g. seawater, PBS-buffer). Ideally, one would filter the actual sample through a 0.02 μm pore-size filter (the majority of the natural phages will pass through larger pore sizes) before fixation and freezing, but often this filtration is difficult and generates substantial unwanted background noise.
6. Add beads as internal standard to the tubes (5 μl of working stock to 500 μl sample; *see* **Note 17**). As these beads have specific characteristics when analysed one can check for variation and errors introduced by the flow cytometer machine. When there is specific interest in the mean green fluorescence of a certain population of phages, normalize the signal to the internal beads.
7. Finally, mix the working stock of SYBR Green I well, spin briefly in a microcentrifuge, and add the dye to a final concentration of 0.5×10^{-4} of the commercial stock (5 μl to 500 μl sample; *see* **Note 18**). Process in dimmed light as SyBR Green I is light sensitive.
8. Incubate the samples at 80°C (for optimal staining characteristics) in the dark for 10 min, after which the samples are allowed to cool in the dark for approximately 5 min before analysis (*see* **Note 19**).
9. Acquisition: Using MilliQ as sheath fluid, run the sample at an event rate below 1000 events sec^{-1} (preferably between 200 and 600 events sec^{-1}), at a flow rate between 20 and 50 μl min^{-1} for 1-2 min. Before starting data acquisition, however, make sure the discriminator is set on green fluorescence and the voltage level is such that no significant electronic noise is generated (*see* **Note 20**). Furthermore, wait for the sample flow rate to stabilize before allowing acquisition of the data; this typically takes about 15 sec, but waiting a little longer will allow a better flush of the flow cell with the sample of interest.
10. Wipe sample needle between each analysis with moist tissue in order to reduce contamination. Change tissue regularly.

11. Analysis: In order to be able to optimally analyze the majority of the particles present one should collect the parameters on logarithmic scales (four-decade dynamic range). Data are collected as list-mode files, which can easily be analysed by a wide range of software (*see* **Note 21**). Viruses are discriminated on the basis of the scatter and fluorescence obtained after staining; green fluorescence *vs*. side-scatter (Figs. 2 and 3). Correct the raw data for the blank (background noise) before calculating the total virus abundance per ml taking into account the dilution factor.
12. When ready with the analysis of the virus samples, make sure the machine is clean by rinsing with BD™ FACSClean and BD™ FACSRinse (generally 10 min each is sufficient), and followed by rinsing with MilliQ (or BD™ FACSFlow).

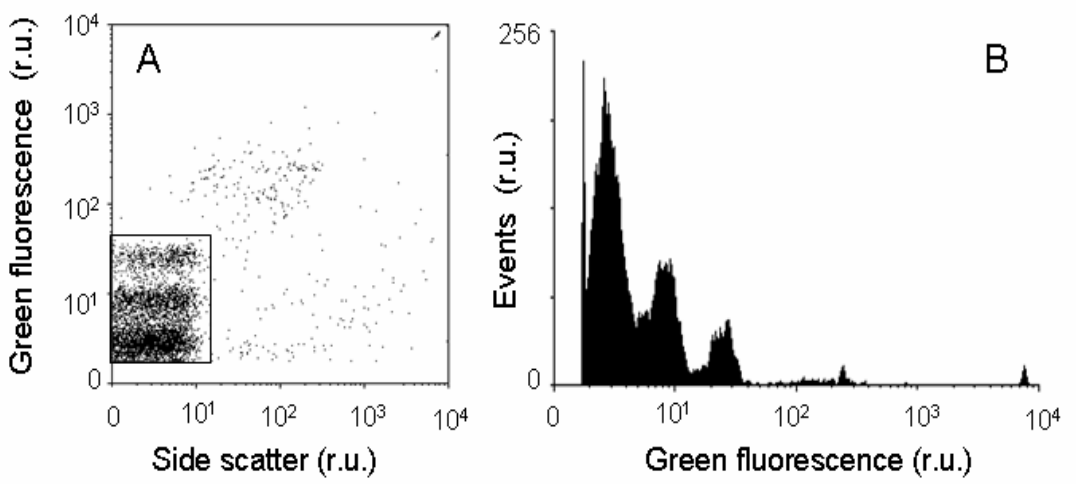

Fig. 2. Typical flow cytometric distribution of a natural seawater sample from the Atlantic Ocean (37° 07' N, 22° 0' W), taken at 70 m depth. (A) Dot plot of side scatter versus green fluorescence. Window represents the total community of natural viruses. (B) Histogram of green fluorescence. The two subpopulations with the lowest fluorescence represent mostly the numerically dominant bacteriophages. The subpopulation with relatively high fluorescence may include algal viruses.

4. Notes

1. Glutaraldehyde should be treated with care, as it is highly toxic. Wear personal protection (gloves, lab coat) and in the hood (good ventilation).
2. Always work in the hood and wear gloves when working with the commercial stock of SYBR Green I as it is dissolved in DMSO, which is a carcinogenic. Trash waste in special container.
3. Unless stated otherwise, all primary and working stock solutions should be prepared in water that has a resistivity of 18.2 MΩ-cm and total organic content of less than five parts per billion. This standard is referred to as "MilliQ" in this text. The water should be freshly prepared.
4. For detection and discrimination of phages, a sensitive flow cytometer is needed. In the present paper reference is made to Becton Dickinson bench top flow cytometers (e.g. FACSCalibur), which was used for the development of the phage enumeration method. This bench top machine

is not too expensive and can be easily transported, which allows enumeration of viruses during field campaigns and/or on board of research vessels (in the case of aquatic virus enumeration).

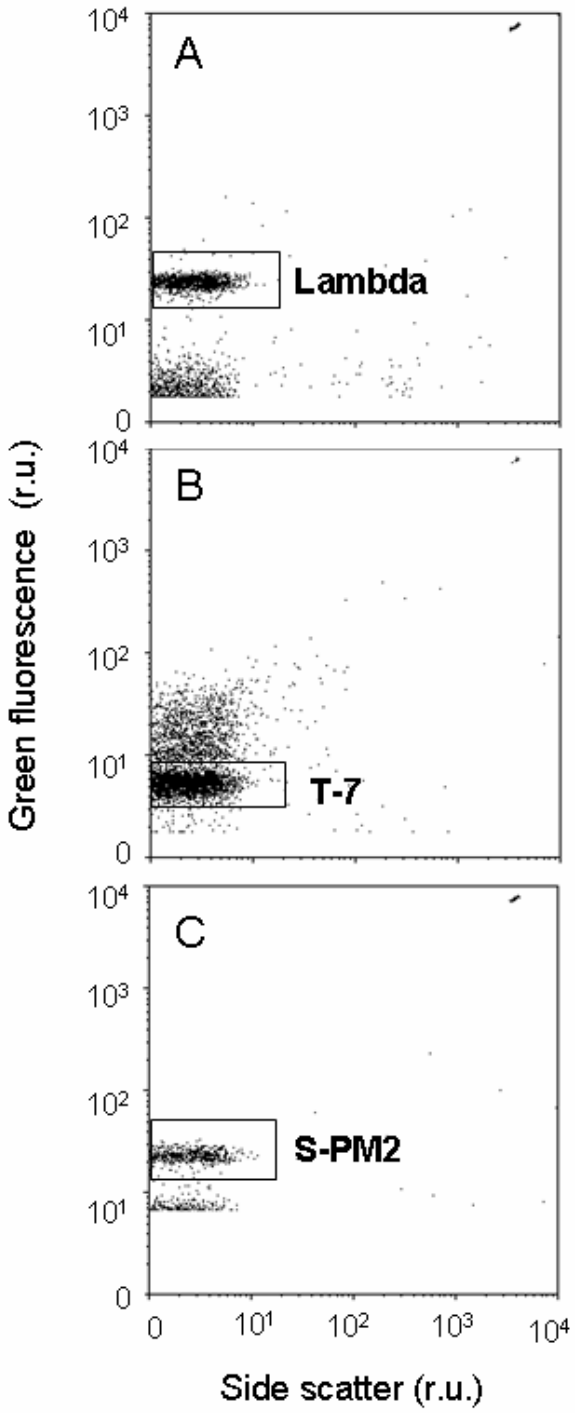

Fig. 3. Histograms of green viral nucleic acid fluorescence of 3 phage species, (A) Coliphage Lambda, (B) Bacteriophage T-7, and (C) photosynthetic marine cyanophage Synechococcus sp. virus (S-PM2). The Bacteriophage T-7 sample showed particles with higher green fluorescence, which represent phage aggregates as could be confirmed using epifluorescence microscopy. The green fluorescence threshold has been raised for the cyanophage S-PM2 sample in order to reduce the number of irrelevant events (other bacteriophages from contaminating bacteria in the culture) and, thus, improve the acquisition of this phage.

5. Standard notation forms are very handy when analysing high numbers of samples and can easily be made in MS-Excel: 14 columns headed ′operator, folder, file name, description, flow rate, time analysis, event rate, gated events total, FSC, SSC, green fluorescence, orange fluorescence, red fluorescence, and trigger′.
6. Staining phage samples with SYBR Green I in combination with flow cytometry provided similar or higher fluorescence intensities and total count as compared to other cyanine dyes (SYBR Green II, SYBR Gold, OliGreen, PicoGreen), making it the most suitable dye for phage enumeration (16).
7. The addition of mild detergents has the potential of facilitating the permeabilisation of the phage particles and subsequently enhancing the green fluorescence. Brussaard (16, 17) tested several ionic and anionic surfactants, such as Triton X-100, Tween 80, NP-40 and SDS, at low final concentrations (0.1% v/v final concentration). Although some improved the virus signal at times, all surfactants generated unsolicited background noise. Detergents should, therefore, be used with care and in addition sufficient blanks should be taken along.
8. It is important to know the accuracy of your virus measurements. Determine the variation in sampling and handling by manifold sampling of the original virus batch and one sample tube, respectively.
9. Fixation has a positive effect on the staining properties of phages as compared to unfixed samples (16). Testing different fixatives at various final concentrations revealed that glutaraldehyde clearly gave the best results. At final concentrations >1% a reduction of total phage abundance was observed. The use of good quality, high purity glutaraldehyde, such as EM-grade, is strongly recommended.
10. The temperature the phage samples are stored at is found to be crucial for reliable enumeration of the phages (12, 16). Storage of fixed, nonfrozen samples results in a rapid loss of phage, in contrast to fixed samples frozen in liquid N_2 and stored at –80°C.
11. To check at which voltage electronic noise is generated, one triggers the appropriate parameter (for virus enumeration Side Scatter and Green Fluorescence are important) and while running a pure solution such as MilliQ, the voltage is slowly increased from zero while keeping an eye on the event rate. The voltage at which the event rate suddenly increases rapidly is the level the PMT starts generating electronic noise. Make sure to set the voltage for checking whether the machine is clean enough somewhat below this critical value.
12. Calibration of the sample flow rate is essential for reliable counts and should be determined regularly during analysis of samples (at least prior and after a day's session of running samples, but preferably more often). Fluorescent microspheres with a known concentration are generally used to estimate the actual flow rate but one should have checked whether this is a valid approach for the sample fluid used as these beads are electrostatic. For example, seawater makes these microspheres sticky, changing the expected concentration. Good results are obtained for the BD FCMs by weighing the sample before and after analysis, which can be extended to other brands of FCMs. In case of relatively large dead-

volumes (e.g. Coulter), a calibration curve should be established in order to determine the actual flow rate. Do realize that seagoing acquisition demands different methods for weighing in not an option at sea; either one does use beads as rough estimate, or one prepares tubes with the appropriate liquid, weighs and seals them, and when on board these tubes are run for a certain time, sealed again to have their weight determined when back on land. A combination of these methods is recommended.

13. Because the abundance of phage declines in the fixed thawed samples it is recommended to analyze only several samples at a time (sets of 8 samples each work well). Upon thawing and preparation of the dilution series, store the samples at 4°C in a fixative fridge, allowing reanalysis within a few hours when needed. Never re-freeze the thawed samples, but trash them after several hours instead. Preferably work in a climate-controlled room (18°C) to avoid warming of the samples by the heat coming from the FCM, and subsequent enhanced loss of phage from the fixed thawed samples.
14. TE-buffer gave the most optimal staining and total count as compared to other choices of dilution solution (water, Tris, PBS, seawater) to dilute the phage samples in (18). Diluted staining reagent SYBR Green I is more stable in buffer than in water, and TE is commonly used in combination with SYBR Green I at a pH of 7-8.5 (Molecular Probes Inc., http://probes.invitrogen.com/products/). As SYBR Green I is pH sensitive, make sure to set the pH of the TE-buffer at a constant value (pH 8.0 gives optimal sensitivity for SYBR Green I).
15. At low dilutions coincidence, i.e. two or more virus particles are simultaneously within the sensing zone of the FCM, becomes a real problem. On average, coincidence occurs for viruses above 800-1000 events sec^{-1}. Working with specific phage species, it is important to determine the optimal event rate because there are certain phages that form doublets or aggregate relatively easily.
16. Previous tests showed that the ratio of TE-buffer to seawater sample could affect the green fluorescence signal and/or the total phage count. Salts can, indeed, profoundly influence the differential absorption values and fluorescence of the complex of SYBR Green and dsDNA . The samples should, therefore, be diluted at least 10-fold and preferably more. In case the original sample contains only a very low total abundance of phage, use a higher flow rate. When working with another type of solutions the phages are suspended in, one should test if and to what extent the ratio sample to buffer influences the phage detection and enumeration.
17. Not having to change the pipette tip every sample, add the beads to the empty tubes before adding the TE-buffer and the sample.
18. Not having to change the pipette tip every sample, add the drop of dye high on the side of the tube and tick the drops down into the TE-buffer carefully when all samples have received the dye. Make sure no phage-containing sample has touched that specific spot of the tube's wall (in doubt, change tips!). Addition of SYBR Green I after heating of the sample results in reduced staining and lower total counts and is, therefore, not recommended.

19. Using lids on the sample tubes is recommended. Use a water bath instead of a heating block to prevent evaporation. Furthermore, when using a lid on the water bath make sure no condense droplets coming from the inside of the lid fall into the tubes.
20. Although you like to have your FCM set as sensitive as possible, you have to make sure the voltage settings for the green fluorescence is ALWAYS set below the level where electronic noise starts to interfere (see for procedure Note 10). The typical setting for the green fluorescence on the BD FacsCalibur FCM in my experience is between 475 and 600. This value is machine- and PMT-dependent and should, therefore, be checked when any manipulation with this PMT has occurred. Typical settings on a FACSCalibur FCM are FSC (forward scatter) = E02, SSC (side scatter) = 550, FL1 (green fluorescence) = 520, FL2 (orange fluorescence) = 500, FL3 (red fluorescence) = 500.
21. In order to allow rapid acquisition of the collected data files using any available software, best is to provide short coding names to the files. A simple, but very efficient filing system is to code the first few characters for the type of parameter analysed, followed by characters for the year month and date. Effective freeware is the programme named ″CytoWin″ (http://www.sb-roscoff.fr/Phyto/index.php), which is especially easy to learn (an advantage when having many students go through the lab).

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Tuesday 25th July

Time	Activity			Location
0900 - 1000	Case study: Keizo Nagasaki **ISOLATION AND CHARACTERISATION OF VIRUSES INFECTING MARINE EUKARYOTIC MICROALGAE**			Resource centre
1000 – 1030	Methods briefings			Resource centre
1030 - 1100	COFFEE			Mess room
1100	Students split into 3 groups (Red, Green & Blue) **TEM: Transmission Electron Microscopy** (Jeol; Keizo Nagasaki; Pete Bond; Roy Moate). NB *This session will be conducted at the University of Plymouth Electron Microscopy Centre* (**UoP**) **Plaque Assays:** (Jim van Etten & Andrea Baker) **Probe Design:** (Declan Schroeder)			MBA and University of Plymouth.
	TEM UoP	**Plaque assays** Room 82	**Probe Design** Resource Centre	
1100 – 1230	Red	Green	Blue	
1230 - 1330	LUNCH			Mess room
1500 - 1530	Green	Blue	Red	
1530 - 1600	COFFEE			Mess room
1530-1700	Blue	Red	Green	
1700 - 1800	POSTER SESSION (Prize for best poster) Wine and nibbles included			Resource Centre
1800	Guest Speaker: John Burden **INSECT VIRUSES – ECOLOGY, BIOTECHNOLOGY AND MANIPULATION IN THE GARDEN.**			Resource Centre
1900 -	DINNER			Mess room

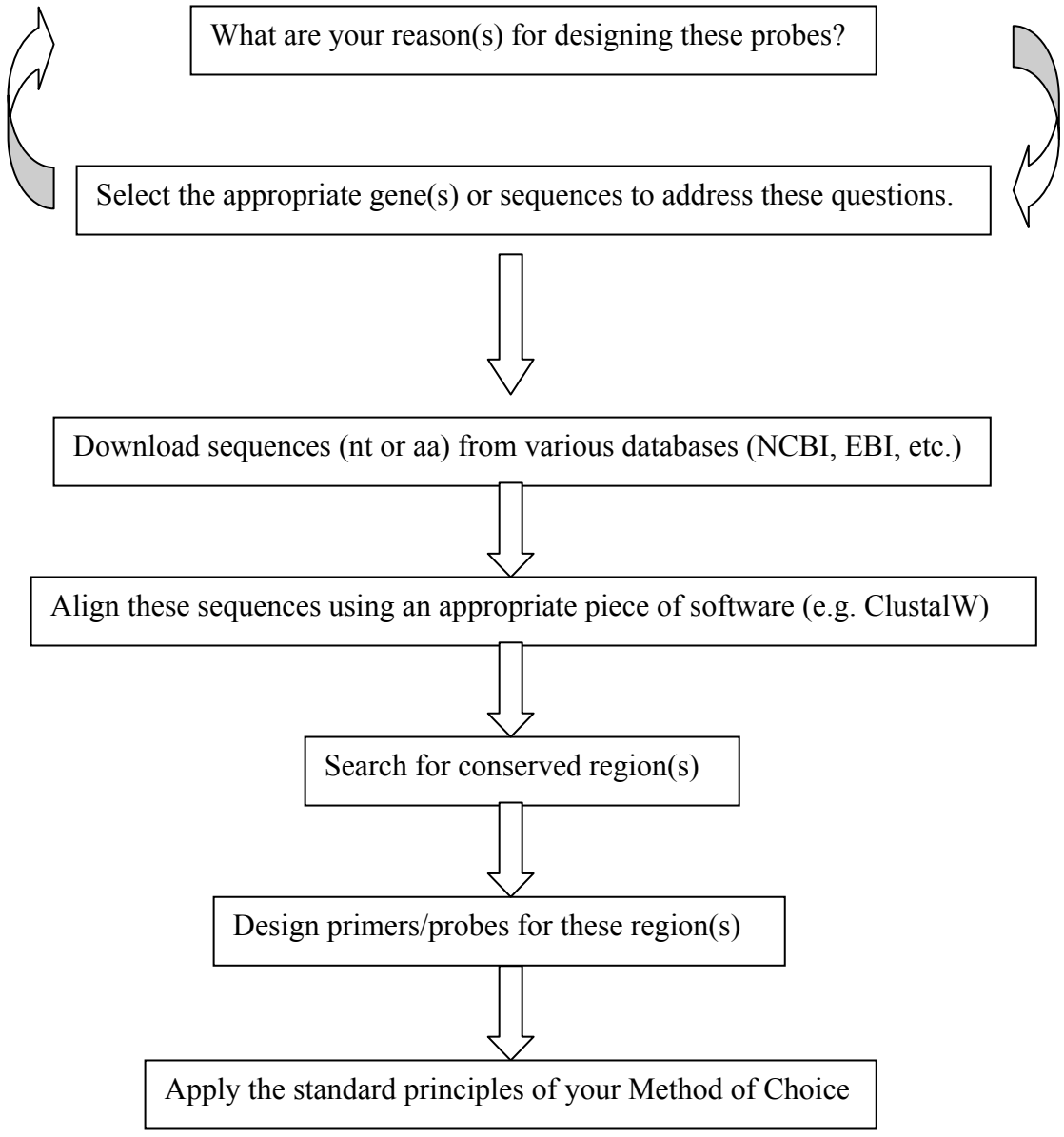
Notes:

PROBE DESIGN:

Declan Schroeder, Nick Bloomer

1. 45 min Lecture 1
2. 15 min Lecture 2
3. 30 min Computer Practical.

Basic flow-diagram on the decision making process when designing molecular probes:



Concepts & Definitions:

Virus: "A molecular genetic parasite that uses cellular systems for its own replication" *Viruses and the Evolution of Life. Luis P. Villarreal.*

- Viruses are real physical entities produced by biological evolution and genetics
- Virus species and higher taxa are abstract concepts produced by rational thought and logic

Virus species: "a virus species is a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche" *8th ICTV Report*

- A "polythetic class" is one whose members have several properties in common, although they do not necessarily all share a single common defining property.

To date (*8th ICTV Report*): 3 Orders, 73 Families (9 Subfamilies), 287 genera, > 1950 species, > 5450 viruses.

- RNA or DNA
- Single (ss) or double (ds) stranded
- Replication strategy: positive sense (+), negative sense (-), Reverse transcriptase (RT), circular, linear, segmented (single or multi-)
- 2000 to 1181000 nts
- Rods to isometric shapes (and almost everything in between) or simply naked
- enveloped or not
- Surface projections or not

Useful web links:

http://www.mcb.uct.ac.za/pcroptim.htm

http://ca.expasy.org/

http://www.ncbi.nlm.nih.gov/

http://www.biodirectory.com/directory/Bioinformatics/PCR_180.html

http://clustalw.genome.jp/

http://www.mbio.ncsu.edu/BioEdit/bioedit.html

Useful references:

Chen & Suttle. Amplification of DNA-polymerase gene fragments from viruses infecting microalgae. *Appl Environ Microbiol.* **62**. 2869 (1996)

Schroeder et al. Coccolithovirus (Phycodnaviridae): Characterisation of a new large dsDNA algal virus that infects *Emiliania huxleyi*. *Arch Virol.* **147**. 1685 (2002)

Culley et al. Metagenomics Analysis of Coastal RNA Virus Communities. *Science.* **312**. 1795 (2006)

Isolation of viruses by plaque assay:

Jim van Etten, Andrea Baker

DESCRIPTION ON WHAT WE WILL BE DOING.

Chlorella viruses

Brief History of the chlorella viruses

Paramecium bursaria chlorella virus (PBCV-1) is the prototype of a rapidly expanding group (family *Phycodnaviridae*, genus *Chlorovirus*) of large, icosahedral, plaque-forming, double-stranded DNA (310 to 370 kb) viruses that replicate in certain unicellular, eukaryotic chlorella-like green algae. In nature, the chlorella host is a hereditary endosymbiont of the cilliated protozoan *Paramecium bursaria* and while inside its protozoan host, the alga is protected from the viruses. The alga host can be grown in the laboratory in liquid and on solid media and since the viruses are lytic to the host, this led to the development of a plaque assay, which was the first virus infecting a photosynthetic host that could be assayed by a plaque assay. Using the plaque assay, chloroviruses have been found in freshwater sources throughout the world and many genetically distinct isolates usually can be found within the same sample. Additionally, the titer of the viruses within a single source can reach as high as 100,000 plaque-forming units per milliliter of water.

Viruses have also been isolated from chlorella symbiotic in the coelenterate *Hydra viridis* and very recently, from the heliozoon *Acanthocystis turfacea*. The symbiotic hydra chlorella have not been cultured and so very little is known about these viruses. However, the *A. turfacea* viruses can be isolated by plaque formation. These viruses are each very specific to their own host isolate; they do not infect nor even attach to the other hosts.

In 1997, the sequence of the PBCV-1 333 kb genome was completed and for several years PBCV-1 was the largest virus genome to be sequenced. PBCV-1 contains ~366 protein encoding genes and a polycistronic gene that encodes 11 tRNAs. The predicted products of ~40% of these genes resemble proteins of known function. Many of the virus genes encode proteins that are completely unexpected for a virus. Often the virus-encoded proteins are either the smallest or among the smallest proteins of their class. Consequently, several virus-encoded proteins are the subject of biochemical and structural studies, including an ATP-dependent DNA ligase, a type II DNA topoisomerase, a prolyl-4-hydoxylase, and a potassium ion channel protein.

Three additional chlorella virus genomes have been sequenced recently and others are nearing completion (see http//greengene.uml.edu). The largest, the 370 kb genome of virus NY-2A, contains ~400 protein encoding genes. Sequence analysis of these additional chlorella viruses indicates that some of the genes encoded by PBCV-1 are not found in other isolates as well as some genes encoded by other isolates are not present in PBCV-1. This suggests that the entire "gene pool" of this genus exceeds the 366 PBCV-1 protein-encoding

genes. Besides their large genome, the chloroviruses have other unusual features including : (i) The viruses encode multiple DNA methyltransferases and DNA site-specific endonucleases; (ii) PBCV-1 encodes at least part, if not the entire machinery to glycosylate its glycoproteins; (iii) PBCV-1 has at least three types of introns : a self splicing intron in a transcription factor-like gene, a splicesomal processed type of intron in its DNA polymerase gene, and a small intron in one of its tRNA genes.

In the following paragraphs, we describe how to plaque assay the chlorella viruses. The ability to plaque assay viruses is an extremely important experimental tool. It allows one to quantify the number of infectious particles in a sample - the samples might be from natural source or one might be studying virus gene expression in the lab. By using the plaque assay to measure infective centers, one can determine the percentage of cells that are actually infected by the virus at any one time. As outlined below, one can also isolate DNA from a single plaque and do PCR on the DNA, e.g. to look for variation in a specific gene.

How to isolate your own Chlorella Virus

- MATERIALS:
  - MBBM plates (or FES plates for Pbi viruses)
  - MBBM soft agar (or FES plates for Pbi viruses)
  - 50°C water bath
  - 50 mM Tris-HCl, pH 7.8, sterile, for dilution blanks
  - 13 x 100 mm tubes, capped and sterile
  - Filter for Sterilization (use Corning cellulose acetate filters - 0.45 micron; for some reason Millipore filters destroy the chlorella viruses).
  - *Chlorella* NC64A, concentrated to 4.0 X 10^8 cells/ml (or Pbi or SAG-241-80)

- PROCEDURE:

1. Take a water sample from a stream, pond or lake
2. Sterilize the water sample through the filter, so that no bacteria etc. will contaminate the agar plate.
3. Pour MBBM plates. Pour the plates either the day of titering or the day before. Fresh plates give more uniform plaque size; old plates (3 days

or more) give more erratic results.

4. Melt the MBBM soft agar and hold in the 50^0C water bath. Dispense 2.5 ml of soft agar to sterile 13 x 100 mm tubes and hold at 50^0C. The soft agar can be made up in quantity in advance and sterilized in 200-250 ml aliquots and stored until ready to use. Melt enough for use. The soft agar can be remelted and used several times.

5. Concentrate the *Chlorella* NC64A to 4.0 X 10^8 cells/ml in the Sorvall centrifuge at 5,000 rpm, 5 min, 4^0C and resuspend the pellets with fresh MBBM. Concentrate enough so that 0.3 ml can be used for each plate.

6. Make up dilution blanks for the virus in the sterile 13 x 100 mm tubes with 50 mM Tris-HCl, pH 7.8. Dilute the virus in 1/10 dilutions.

7. Label the plates.

8. Titering. Remove 13 x 100 mm tubes of soft agar from the water bath to a test tube rack as needed. To each tube, add 0.3 ml of the concentrated chlorella and 0.1 ml of the diluted virus (when tittering viruses that grow slower, use 0.05 to 0.1 ml of the concentrated chlorella instead of 0.3 ml). Mix briefly (by rolling the tubes between the palms of the hands) and pour the contents of the tube onto the plate. Tilt the plate gently until the entire surface of the plate is covered with soft agar (this needs to be done quickly, as the soft agar will solidify quickly once it has been poured onto the plate). Allow the agar to solidlfy (a few minutes), invert the plates with the lid down (so moisture condensation stays in the lid and off the agar surface), and incubate the plates at 25^0C in continuous low light. Stack the plates only two deep, as the plaque size will increase if the plates are stacked deeper. Plaques will be ready to count after 3-4 days incubation. However, you can actually begin to see plaques by 24 to 32 hr.

9. Use MBBM plates and soft agar for titering viruses with SAG 241-80 chlorella and FES plates and soft agar for titering viruses with Pbi chlorella.

MBBM :

To 950 ml of distilled water add:
10 ml of stock solutions 1-6
1 ml of stock solutions 7-9
2 ml of stock solution 10
1 g bacto-peptone
5 g sucrose

Tetracycline (filter sterilized, 10 mg/ml final conc) is added after the media is autoclaved and cool.

For MBBM plates, agar is added to 1.5% before autoclaving. For MBBM soft agar, agar is added to 0.75% before autoclaving.

Stock solutions:

1. 25 g NaNO3 per liter d-H_2O
2. 2.5 g CaCl2*2H2O per liter d-H_2O
3. 7.5 g MgSO4*7H2O per liter d-H_2O
4. 7.5 g K2HPO4 per liter d-H_2O
5. 17.5 g KH2PO4 per liter d-H_2O
6. 2.5 g NaCl per liter d-H_2O
7. 50 g disodium EDTA, 31 g KOH per liter d-H_2O
8. 4.98 g $FeSO4*7H_2O$ per liter acidified H_2O (Acidified H_2O is 999 ml d-H_2O + 1 ml conc. H_2SO_4)
9. 11.42 g H3BO3 per liter d-H2O H_2O
10. 8.82 g $ZnSO_4*7H_2O$, 1.44 g $MnCl_2*4H_2O$, 0.71 g MoO_3, 1.57 g $CuSO_4*5H_2O$, and 0.49 g $CoNO_3*6H_2O$ per liter d-H_2O

PCR from Plaques

The following method is how we isolate the a chlorella virus gene that encodes a potassium ion channel protein from a single plaque. We are interested in this particular gene because it encodes the smallest protein (94 amino acids) known to form a functional two-transmembrane, one pore potassium ion channel. Furthermore, homologous genes from different chlorella viruses encode proteins with amino acid substitutions in the protein and some of these amino acid substitutions lead to potassium ion channels with different properties. Consequently, we are screening chlorella viruses from native sources for diversity in this particular gene as well as some other genes.

Primers for the potassium ion channel (Kcv)

PBCV-1 Kcv 5' primer BamHI
CGG GGA TCC ATG TTA GTG TTT AGT AAA

PBCV-1 Kcv 3' primer XhoI
TCT CTC GAG TCA TAA AGT TAG AAC GAT

Procedures

1. Pick up a single chlorella virus plaque from the lawn with a wide bore pipette tip (cut the regular pipette tip at the 1.5 cm from the tip, and sterile to get 'wide bore pipette tip'), put to a 1.5 ml microfuge tube, smash the single plaque with tooth picks, pipette tips or other sterile tools, add 100 ul 50 mM TrisCl, pH 7.8, vortex for 1 min, brief spin (6,000 rpm, 2 min by benchtop centrifuge)

2. Move 30 ul of the supernatant to a clean microfuge tube, boil 10 min to disrupt the virion particles and denature the double stranded DNAs, chill on ice as PCR template.

3. For KOD protocol, in total 50 ul volume please add:

template	5 ul
5' primer(5 pmol/ul)	3 ul
3' primer(5 pmol/ul)	3 ul
4xdNTPs	5 ul
10xPCR buffer	5 ul
$MgSO_4$	2 ul
KOD DNA polymerase	1 ul
H_2O	26 ul
Total	50 ul

Cycles: 94 C for 2 min, then 94 C for 15 sec, 60 C for 30 sec, 68 C for 60 sec (for each Kb) for 30 cycles, finally 68C for 10 min.

4. For Vent or other DNA polymerases, in total 50 ul volume add:

template	5 ul
5' primer(100 pmol/ul)	1 ul
3' primer(100 pmol/ul)	1 ul
4x dNTPs(10 mM each)	1 ul
10x thermopol buffer	5 ul
($MgCl_2$ included already, if not, add Mg to final conc 1 mM)	
Vent DNA polymerase	1 ul
H_2O	39 ul

Web sites:

1. http://greengene.uml.edu/ The GreenGene Server was developed for DNA or protein sequence analysis in Dr. Michael V. Graves' laboratory in the Department of Biological Sciences, University of Massachusetts-Lowell. Currently, the GreenGene server provides a local database containing the genomes for several chlorella viruses (family Phycodnaviridae, genus Chlorovirus), and four types of analysis tools: 1) BLAST searching both the NCBI and local databases in a batch fashion. 2) Parsing the BLAST results. 3) Predicting open reading frames. 4) Generating files for genome annotation.

2
. GiantVirus.org, the giant virus site, home of Mimivirus and other large DNA viruses

Practical Session-

A) Preparing Plaque Assays

NB: MBBM plates and aliquots of soft agar have been prepared in advance and water has previously been filtered due to time constraints.

In pairs

1. Starting with a flask of *Chlorella* NC64A (approximately containing 100 ml culture), split the culture into 2 x 50 ml Falcon tubes (label these with your name) and make sure each tube contains the same volume.

2. Concentrate the cells by centrifugation in the bench top centrifuge for 5 minutes at 5,000 rpm (4°C). Ask for assistance before using the centrifuge.

3. After centrifugation, carefully remove the tubes from the centrifuge so not to disturb the pelleted cells.

4. Carefully pour off the supernatant, leaving the pellet and a small amount of overlying media.

5. Resuspend the *Chlorella* cells in approximately 2 ml fresh MBBM media.

6. Prepare 1 in 10 dilution series of the virus inoculum (the water sample) in eppendorfs using 50 mM Tris-HCl in 1 ml volumes.
   i.e. 100 µl water sample + 900 µl Tris = 10^{-1}
   100 µl 10^{-1} dilution + 900 µl Tris = 10^{-2} and so on to 10^{-5}

7. Take 100 µl of each dilution and also 100 µl Tris for a control and place into a new eppendorf (make sure they are labeled).

8. To each virus aliquot, add 300 µl concentrated *Chlorella* cells.

9. Before the next step make sure your MBBM plates are labeled with your name and also the dilution. 6 plates between each pair.

10. 2.5 ml MBBM soft agar has been previously aliquoted into universals which are in the 50 °C water bath.

11. Remove tubes of the soft agar from the water bath.

12. To each of the tubes add the *Chlorella* virus suspension (approx 400 µl).

13. Mix briefly, by rolling the tubes between the palms of the hands and pour the entire contents onto the surface of the plates.

14. Quickly, tilt the plate so that it is completely covered with the soft agar.

15. Allow the agar to solidify and then invert the plates with the lid facing down.

16. Incubate plates at 25 °C in continuous light. You can check your plates later in the week!

B) Picking plaques

1. Using the pre-prepared plaque assays practice picking plaques.

2. Using a sterile Pasteur pipette, pick a plaque from the lawn by stabbing the tip (gently) directly into the centre of the plaque.

3. Transfer the plug of agar to a sterile eppendorf. This can then be used for PCR or could be resuspended in Tris and used in later experiments such as liquid lysis, TEM or further virus purification.

C) Calculating the virus titre

1. Use one of the pre-prepared plates or one in the pictures, to practice calculating the concentration of the initial virus suspension in plaque forming units (PFU)/ml.

2. Count the number of plaques on a plate which has well separated plaques, the use the following equation.

Number of plaques x Dilution factor* x 10* = Concentration in PFU/ml

* Dilution factor- If counting 10^{-2} plate, multiply by 100

* x 10 - This is used as you only plated with 0.1 ml virus and you want PFU/ml.

Electron microscopy

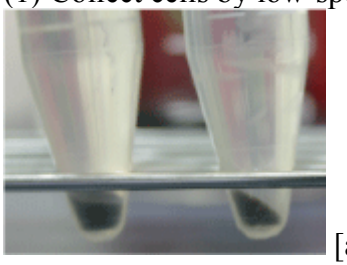
Expert: Keizo Nagasaki, Pete Bond, Andy Yarwood
Demonstrators: Willie Wilson, Matt Hall

[HOW TO PREPARE VIRUS SAMPLES FOR TEM OBSERVATION]

A: Thin-sectioned sample

1. Fixation

(1) Collect cells by low-speed centrifugation (1200-1500 rpm) [a]



[a]

(2) Discard the supernatant slowly [b]

[b]

(3) Add 3ml of the 1st fixative (1-2% glutaraldehyde in 0.1µm-filtered SW): Be sure to observe the fixed cells under light microscope. If cells collapse at this procedure, please try the method given by Hara & Chihara [Jap J Phycol **30:** 47-56 (1982)]. As for the other promising procedures, please refer the following articles. Especially, raphidophytes are very difficult to fix in a good condition.

- Inoue et al. Jpn J Phycol **40:** 333-348 (1992)
- Yokote et al. Mar Biol **88:** 295-299 (1985)
- Wada et al. Protoplasma **137:** 134-139 (1987)
- Cattoloco et al. Plant Physiol **57:** 497-503 (1976)
- Noro & Nozawa. Jap J Phycol **29:** 71-78 (1981)

(4) Keep the fixed samples in refrigerator (>3hr)

(5) Wash with 10ml 0.1M PB (pH7.2-7.4) twice

(6) Add 1ml of the 2nd fixative (2% OsO_4 in 0.1M PB): OsO_4 is most dangerous reagent used in the procedures. [c]

http://blink-prod.ucsd.edu/Blink/External/Topics/How_To/0,1260,15753,00.html

[c]

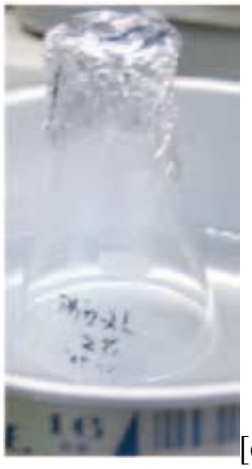
(7) Tap or voltex to break up the pellet [d] and then keep in refrigerator (this should be permitted to use for OsO_4) for 3-4 hrs (It is recommended to avoid over-fixation)

 [d]

(8) Washing with 10ml 0.1M PB (pH7.2-7.4) 2-3 times

2. Preparation of tissue

(1) Suspend the resultant pellet with 1% low melting-temperature agarose solution (at 70-90 C [e] [f]

 [e]  [f]  [g]

(2) Pour the cell suspension in agarose solution into a microtube (ex. Eppendorf) [g]

(3) Immediately centrifuge at 12000 rpm for 2 min then cool the tube in refrigerator [h]

 [h]

Hollow out the agarose-cell-pellet [i] and cut the material into 1.0-1.5 mm^2 blocks using appropriately split razor pieces. [j,k,l]


[i]

[j]

[k]

[l]

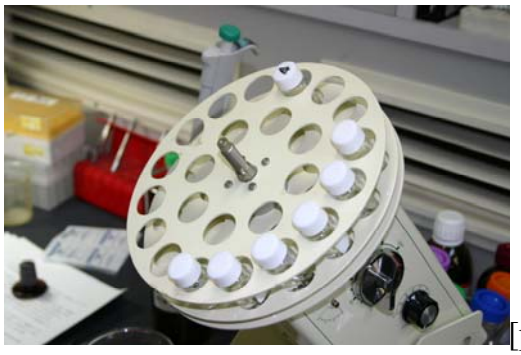
3. Dehydration and infiltration

(1) Dehydrate the blocks with the following concentrations of ethanol in a graded ethanol series (3-5 minutes per change): 50%, 70%, 80%, 90%, 95%, 98%, 99.5% (Numbers marked on the bottle is covered with white tape to prevent fading.) [m,n]


[m]

[n]

(2) Be careful not to dry up the blocks at any procedure [o] (Rotate slowly the bottles [p])


[o]

[p]

(3) Continue dehydration with two more changes of 100% EtOH for 10 minutes per change (Note: To prepare 100% EtOH, add molecular sieve into 99.5% EtOH and leave it for a few days [q])


[q]

(4) Replace the 100% EtOH with three times of 20-30 minutes change of propylene oxide (PO). Use PO in the fume hood. (Be careful not to dry up the blocks at any procedure)

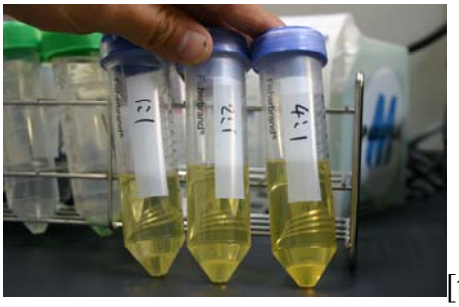
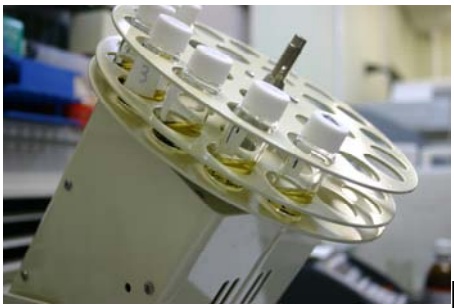
(5) Infiltrate the blocks using the following concentrations of appropriate resin*, approximately >5 hrs per change: (1:1) resin : PO, → (2:1) → (4:1)

* We generally use Quetol 653 resin. [r,s,t,u]

Kushida, H. An improved embedding method using ERL 4206 and Quetol 653. J Electron Microsc **29**: 193-194 (1980).


[r]

[s]

 [t]  [u]

(6) Infiltrate the blocks using the 100% resin for >12 hrs

(7) Embed in fresh resin using the molds (Copolymerization with a numbered paper is very usuful) [v,w]

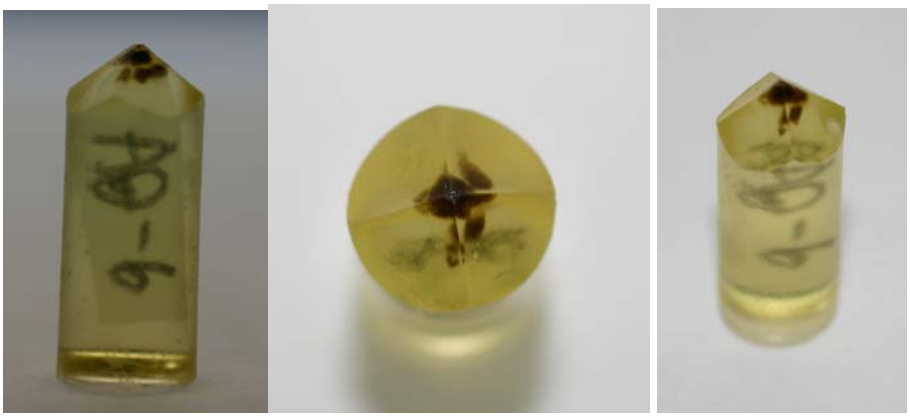
 [v]

 [w]

(8) Polymerize for 24 to 48 hrs at an appropriate temperature (60 C) [x]

 [x]

Now you've got your block samples!



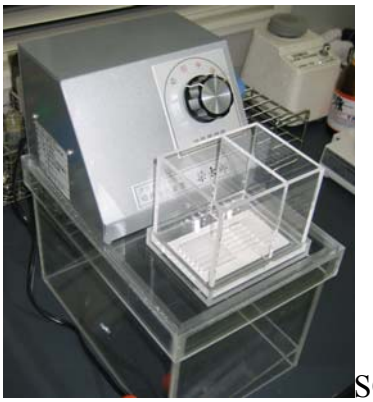
4. Thin-sectioning and electron staining

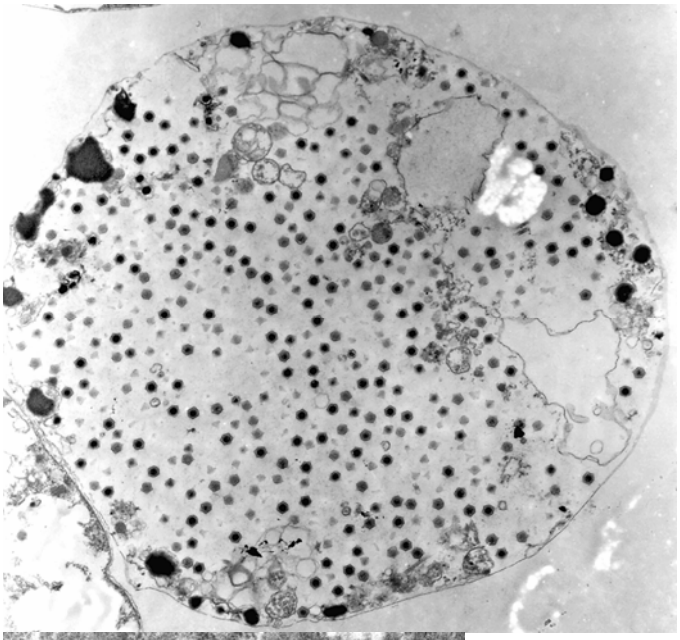
Refer excellent websites as given below:

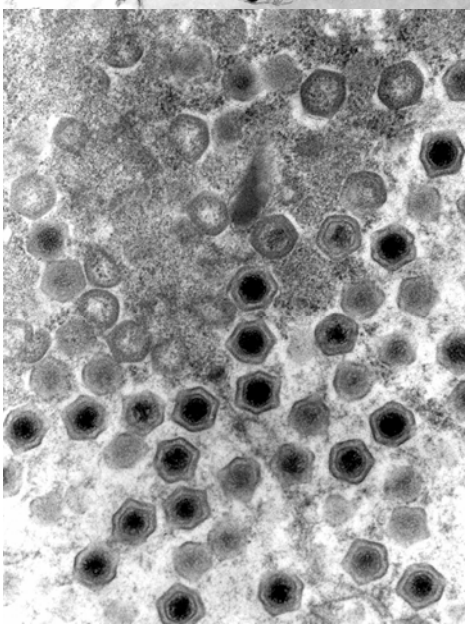
http://www3.utsouthwestern.edu/mcif/ThinSectioning.htm
http://synapses.mcg.edu/lab/howto/protocols/thinsectioning.htm
http://synapses.mcg.edu/lab/howto/protocols/em_index.html
http://biovisa.net/protocol/protocol_read.php3?pid=2805

[Tips]

- Distinguish your diamond knives to use appropriately: for cutting "culture samples" and "natural field samples". Note that natural samples always contain small fine grains of sand or mud, which easily degrades the diamond edge.
- Using clean ultrapure water in thin-sectioning process is strongly recommended.
- Do not hurry up. Note that this is the final stage of TEM-sample preparation.
- Use of a staining device "SOMETARO" is strongly recommended.


SOMETARO- staining device





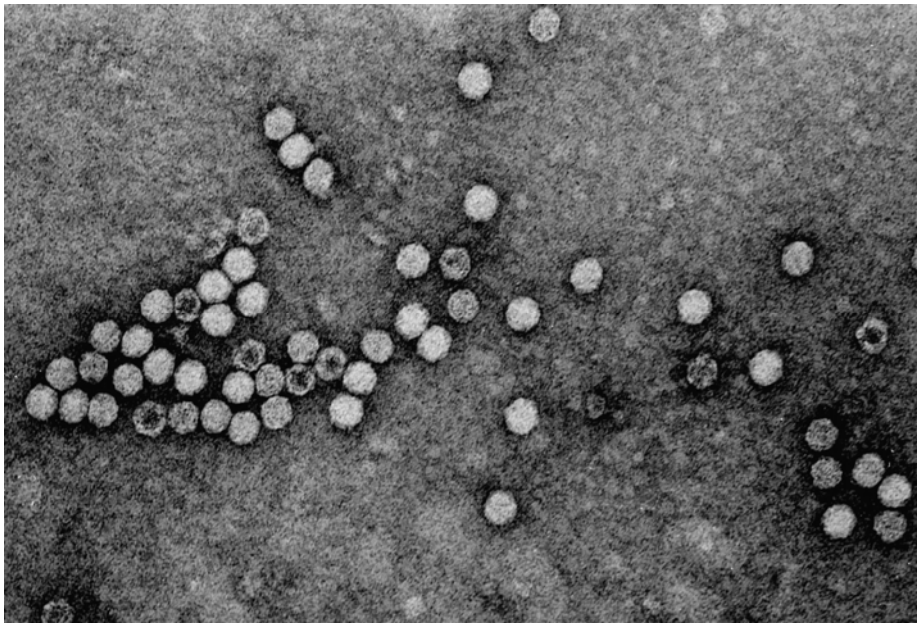
Thin-section of algal cell infected with HcV (*Heterocapsa circularisquama* DNA virus)

B. Negative-staining technique

Refer excellent websites as given below:

http://biovisa.net/protocol/protocol_read.php3?pid=2805

http://www2.mrc-lmb.cam.ac.uk/groups/hmm/techniqs/Negative%20stain%20protocol.htm



Negatively-stained RsRNAV (*Rhizosolenia setigera* RNA virus) particles

Directed by
Keizo Nagasaki & Yuji Tomaru
Harmful Algal Bloom Division,
National Research Institute of Fisheries and Environment of Inland Sea,
Fisheries Research Agency, 2-17-5 Maruishi, Hatsukaichi, Hiroshima 739-0452,
Japan, Tel: 81 829 55 3529 Fax: 81 829 54 1216 E-mail: nagasaki@affrc.go.jp

Negative staining of bacteria and viruses for observation in the transmission electron microscope.

Negative stains differ from positive stains in that they do not react with the specimen at specific binding sites, instead they accumulate on and around the specimen. Areas where stain accumulates are more electron opaque than regions where the stain is more dispersed. Consequently, particulate material such as bacteria and viruses around which stain builds up are effectively highlighted from the substrate. Features such as bacterial cell wall architecture and viral microstructure will collect stain and become visible in the microscope (Fig. 1).

Figure 1. Diagrammatic representation of heavy metal negative stain accumulation on and around a bacterium.

The most popular stains are ammonium molybdate, phosphotungstic acid and uranyl acetate - recipes are described below.

Staining methods are very straightforward (see below) but you will need to determine the most appropriate concentration of bacteria or virus using a standard dilution series. Similarly, the duration of staining will need to be optimised for your particular samples.

If your images are not adequate then consider where you may have gone wrong with respect to the staining method, taking into account your theoretical knowledge of the negative staining procedure. Pay attention to your practical method and its accuracy; stain concentration, pH and time of staining etc. and the dilution series of your samples. From your images determine the most likely causes for poor quality. Go back and repeat the experiment taking this into account.

AMMONIUM MOLYBDATE

1. Prepare a 2% aqueous solution of ammonium molybdate.

2. Adjust to pH7 with 1.0 M KOH.

3. Add 10mg bovine serum albumen (BSA) as wetting agent if required, (may improve staining qualities).

PHOSPHOTUNGSTIC ACID (PTA)

1. Prepare a 2% aqueous solution of PTA.

2. Adjust pH to between 6.8 and 7.4 by adding drops of 1.0 M KOH.

3. Add 10 mg bovine serum albumen as wetting agent if required.

URANYL ACETATE

1. Prepare a 2% aqueous solution of uranyl aceteate.

2. Adjust pH to between 2 and 4.5 with 1.0 M HCl or KOH

3. Add 10mg bovine serum albumen as wetting agent if required.

STAINING METHODS:

Method 1.

1. Place a 30µl drop of suspension of material to be stained on a wax sheet, place a coated grid on to the drop and leave for 30-60 seconds.

2. After appropriate time (15-60 seconds) blot the grid by pressing one edge down on to filter paper.

3. Place the grid on to a drop of 2% uranyl acetate for 5-10 seconds, blot dry and immediately place the grid on to a second drop of 2% uranyl acetate for 30-60 seconds, blot dry and observe in the TEM.

Method 2.

1. Suspend material directly in the stain and apply to the coated grid as above.

Method 3.

1. Mix one drop of stain with one drop of suspension and apply to the coated grid.

Concentration is important:

Too much material displaces the stain or piles up forming poorly stained areas.

Too little material means much time is spent searching in the microscope for specimens.

Thursday 27th July

Time	Activity			Location
0900 - 1000	Case study: Jim van Etten **CHLORELLA VIRUSES**			Resource centre
1000 – 1030	Methods briefings			Resource centre
1030 - 1100	COFFEE			Mess room
1100	Students split into 3 groups (Red, Green & Blue) **Nuc Ac Extn:** Nucleic Acid Extraction (John Paul & Mike Allen). **Productivity:** Dilution experiments to determine virus productivity (Markus Weinbauer & Susan Kimmance) **QPCR:** Quantitative PCR (Techne)			MBA
	Nuc Ac Extn: Room 5	**Productivity** Resource Centre	**QPCR:** Room 82	
1100 – 1230	Red	Green	Blue	
1230 - 1330	LUNCH			Mess room
1500 - 1530	Green	Blue	Red	
1530 - 1600	COFFEE			Mess room
1530-1700	Blue	Red	Green	
1700 - 1730	POSTER SESSION (Prize for best poster) Wine and nibbles included			Resource Centre
1730	Guest Speaker: Nick Mann **EVOLUTIONARY PRESSURES ON MARINE VIRUSES**			Resource Centre
1900 -	Plymouth Gin Distillery Tour			Plymouth Gin Distillery
2015	DINNER			Plymouth Gin Distillery

Notes:

Thursday 27th July

Nucleic acid extraction techniques

John Paul, Mike Allen

Viruses are incredibly diverse and this is reflected in their genomes which can be composed of RNA or DNA in a single or double stranded and linear, circular or segmented form. The smallest viruses have only a handful of genes and tiny genomes (measured in 1000s of bases), whereas the largest may contain many hundred of genes and could have over a million bases. This makes it very difficult to write a definitive guide to virus nucleic acid extraction. Indeed, not only do you have to deal with isolating genomic material from purified viruses, but you may also need to extract RNA transcript from their hosts during an infection cycle as well. This may mean using a completely different extraction technique.

All of these variables will need to be taken into account when you choose your extraction technique. One of the most important things to note is the major difference in stability between RNA and DNA. DNA is very stable (chemically and biologically), however RNA is not! The extra hydroxyl group in the ribose ring in RNA makes RNA chemically less stable than DNA, but also the presence of contaminating RNAses often results in RNA degradation. Modern RNA extraction kits (Qiagen RNeasy and Stratagene Absolutely RNA) essentially eliminate many complications of working with RNA. However, highly purified RNA does not store well under any conditions and usually must be stabilized by a chaeotropic agent such as guanidinium HCl or isothiocyanate.

A major issue with virus samples is the biomass which is typically limited to relatively small yields. This is not as big a problem as you may think it is, there are some excellent methods of amplifying your genomic message such as Amersham's Genomiphi, Sigma's GenomePlex and Qiagen's RepliG. However, these can induce sequence artifacts that are often difficult to detect or weed out. Depending on the down stream application it may be more important to have a clean but low yield preparation than a dirty, high yield preparation.

Often phage lysates from pure cultures are at a high enough titer compared to contaminating host DNA that a commercial kit can yield highly purified viral DNA at sufficient concentration for direct sequencing. Other viral lysates (ie. Particularly those of temperate phages from prophage inductions and especially ambient phage communities) need to have the phage particles purified by CsCl banding (ultracentrifugation) to separate phages from dissolved DNA and other schmutz. This purification of viral particles of course precedes any nucleic acid extraction.

The basis of any extraction is to separate what you want from what you don't want. For viruses, this is started by breaking open the capsid. This can be done either enzymatically (with an enzyme such as Proteinase K), with detergents (such as SDS) or a combination of the two. Once you have disrupted the cells (or in the case of viruses the virion capsid) the nucleic acids must then be

separated from any proteins, lipids and other cellular junk. By increasing the ionic strength (using high salt solutions) you can disrupt the interaction between proteins and nucleic acids, freeing up the nucleic acids in solution. Phenol and chloroform can also be used to dissociate nucleic acids from proteins (it also dissociates some lipids and polysaccharides). Genomic nucleic acids are large molecules (long strings of nucleotides) and tend to precipitate out of solution when their surrounding water is removed. Thus, by adding ethanol during extraction you can separate the nucleic acids away from the cellular debris.

Commercial kits avoid the use of phenol and chloroform and typically use small chromatography columns that take advantage of the ability of DNA to bind tightly to powdered glass under high salt conditions. The glass can then be washed to remove contaminants, and the pure DNA is then eluted with a low ionic strength buffer.

The most important aspect of doing any nucleic acid extraction method is the starting material. Samples that are more concentrated, purified and cleaner will always give better yields of higher quality.

Remember (!):

Rubbish in = Rubbish out

Translation: sometimes it is better to take the extra effort to CsCl purify your viruses prior to nucleic acid extraction!

Nucleic Acid Extraction Kits

The following companies make commercially available kits for nucleic acid extraction. Depending on the nature of the source material you may need to shop around to find a kit suitable for your requirement. When extracting RNA for transcriptomic analysis the nature of the virus' host will dominate your selection of kit. For genomic preps many companies make genomic RNA and DNA specific extraction kits. Most kits come with extensive instructions for adapting protocols to suit your purpose, but if you have any doubts about what can and can't be done, we recommend you contact their technical resource people for advice. These companies are fiercely competitive and will make sure they can solve your problems in order to get the sale! Make sure you ask for sample kits to test that their protocols work before you buy in bulk!

Promega	http://www.promega.com/
Ambion	http://www.ambion.com/
Qiagen	http://www1.qiagen.com/
ABgene	http://www.abgene.com/
Invitrogen	http://www.invitrogen.com/
Sigma	http://www.sigmaaldrich.com/
Bio-Rad	http://www.bio-rad.com/
Roche	https://www.roche-applied-science.com/
Stratagene	http://www.stratagene.com/

CsCl Banding of Phage Preps

Cesium Chloride gradient purification for marine phages (modified from Sambrook)

A day before:

I. Prepare a 0.02 µm filtered 75% Artificial Seawater to make gradients

II. Prepare 3 cesium chloride solutions with different densities:

Density (p) (g/ml)	CsCl (g)	75% ASW (ml)
1.3	39	85
1.5	67	82
1.7	95	75

a. Make gradients a little denser in case they need to be modified

b. Allow solutions to equilibrate overnight

c. Use hydrometer to measure density of each solution; adjust density with 75% ASW in necessary.

1. Measure volume of phage suspension and add 0.5 g CsCl per ml. Mix gently to dissolve.

2. Make step gradients by layering 2.2 ml of the prepared CsCl solutions of increasing density under one another in Beckmann Polyallomer centrifuge tube. This can be easily done using blunt end stainless steel pipetting needles. Use a Sharpie to mark the interface between each layer on the outside of the tube.

3. After layering the gradients, add 4.4 ml of phage suspension to the top. Make sure to make a mark every 2.2 ml.

4. Centrifuge the gradients in an ultracentrifuge (Beckman LE-80 or equivalent) at 29,000 rpm for 2 hours at 4° C using an SW40Ti rotor. Be sure to balance the tubes by weight and load the rotor properly.

5. After centrifugation, use a ring stand assembly to securely hold the sample tube.

5. Using a 20 gauge needle to bore a hole near the bottom of the tube. Collect the following fractions: 1.7, 1.5, 1.5-1.3 interface, and 1.3 based on the marks on the tubes. The phage particles should be at the interface between the 1.3 p and 1.5 p gradients. There may be a white debris band in the 1.3 gradient.

6. Prepare Sybr Gold slides of each fraction to enumerate virus particles. There should be around a 25 percent loss of virus particles from the PEG precipitation.

7. Proceed to extraction of phage DNA.

Nucleic acid extraction on the cheap (not kit based)

The following protocol has been used to purify the genomic DNA from coccolithoviruses from lysed seawater cultures (filtered through a 0.45 µm filter).

1. The PEG and CTAB protocol (From Willie Wilson's Laboratory)

PEG (to concentrate the virus fraction)

1) Add 58.4 g/l NaCl & dissolve gently. Let it stand on ice or in fridge for at least an hour.
2) Centrifuge for 10 minutes at 5000 rpm and retain supernatant.
3) Add PEG6000 for a final percentage of 10% & dissolve gently. Let it stand on ice or in the fridge over night or for at least two hours. **The longer you let it stand at the cool temperature the better the yield.**
4) Centrifuge at 6000 rpm for 25 minutes. Discard the supernatant and invert the tubes on paper towels for a few minutes.
5) Resuspend the pellet in a suitable buffer (phage buffer/ phosphate buffer/ tris buffer) and store at 4 °C or proceed to CTAB protocol if you require DNA.

CTAB (to extract DNA)

1) Add 500 µl of prewarmed lysis buffer (0.5% SDS, 20 µg/ml proteinase K) to pellet and vortex 30 seconds.
2) Incubate at 55 °C for 30 minutes (mix every 10 minutes).
3) Add 80 µl 5M NaCl and 100 µl prewarmed CTAB solution (10% CTAB (hexadecyltrimethyl ammonium bromide) in 0.7M NaCl. Dissolve on heated stirrer at 65 °C).
4) Incubate at 65 °C for 10 minutes.

5) Add 500 µl Chloroform:Isoamyl alcohol (24:1) and mix well.
6) Centrifuge at full speed (14,000 rpm) for 5 minutes.
7) Remove top phase to new tube and add 360 µl (0.6 volumes) of Isopropanol.
8) Mix contents by inverting tube gently a few times.
9) Leave tube at room temperature for 10 minutes to allow a precipitate to settle out.
10) Pellet DNA by centrifugation at full speed (14,000 rpm) for 10 minutes.
11) Wash pellet in 500 µl cold 70% ethanol (spin for 5 minutes).
12) Air dry pellet and resuspend in 35 µl TE.

2. Formamide extraction of phage DNA (from John Paul's Laboratory)

1) If purified lysate using cesium chloride need to get rid of the salts that may interfere with DNA extraction. Dialyze lysate against 1500 ml TE buffer for 24 hours, change buffer 3 times.
2) Add the following to the lysate:

   0.1 volumes 2 M Tris HCl

   0.5 volumes 0.5 M EDTA

   1.0 volume formamide (molecular biology grade)

   2 µl glycogen per ml sample.
3) Heat sample at 65° C for 30 minutes
4) Precipitate DNA with 6 volumes of ice cold EtOH for 30 min at -80° C or overnight at -20°C.
5) Pellet sample for 10 minutes at 10,000 g.
6) Redissolve pellet in 300 µl TE.
7) Precipitate DNA with 6 µl 5 M NaCl and 750 µl EtOH for 30 min at -80° C.
8) Pellet sample for 10 minutes at 10,000 g.
9) Resuspend pellet in 300 µl TE, do not pipette.

3. Phenol/chloroform DNA extraction (from Willie Wilson's Laboratory)

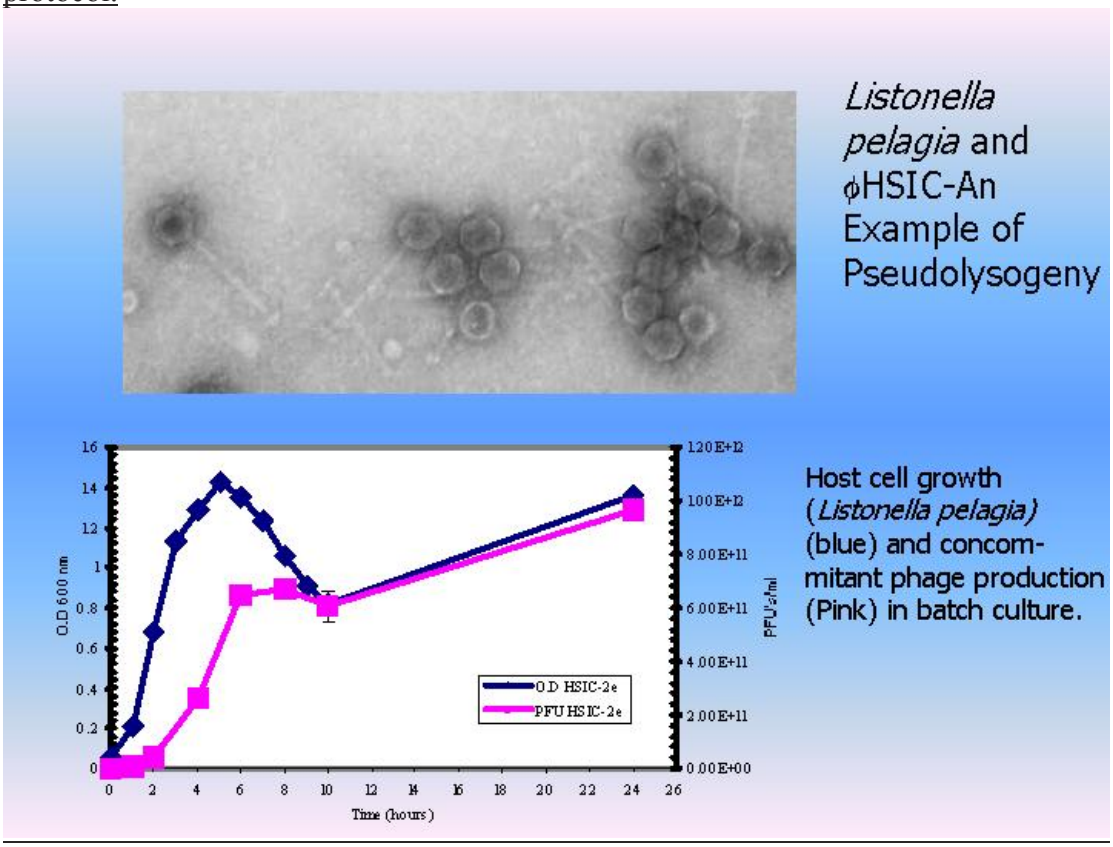
1. Add 1/5 volume of TE buffer to the purified virus solution.
2. Incubate at room temperature for 30 mins.
3. Add 1 volume of water-saturated phenol and vortex gently.
4. Centrifuge at full speed (14,000 rpm) in a benchtop centrifuge for 30s.
5. Remove the TOP, water phase containing the DNA into a clean tube.
6. Add 1 volume of chloroform and vortex briefly again.
7. Spin briefly.
8. Remove the TOP water phase into a clean tube.

Quick and cheap. But beware: phenol is a nasty chemical to work with!

Today's Demonstration: The Wizard® Lambda Preps DNA Purification System using the Pseudotemperate phage ϕHSIC

The Wizard® Lambda Preps DNA Purification System is a fast, reliable method for
the purification of lambda DNA from plate or liquid culture lysate. The entire procedure can be completed in 1.5 hours or less with no organic extractions or ethanol precipitations. The lambda DNA is eluted from the Wizard® Lambda DNA Purification Resin in water or TE buffer. Lambda DNA purified using Wizard® Lambda Preps System is of high purity and may be used in restriction digestions, ligations and sequencing without any further purification. For convenience and efficiency, multiple samples may be processed at one time using a vacuum manifold such as the Promega Vac-Man® or Vac-Man® Jr. Laboratory Vacuum Manifolds.

The phage you will be using today is ϕHSIC, a pseudotemperate member of the *Siphoviridae* that infects *Listonella pelagia* (formerly *Vibrio pelagius*; Jiang et al., 1998; Williamson et al., 2001). This phage can establish a pseudolysogenic relationship in that cultures harbouring the phage yield high titers of phage (>10^{11}/ml) while producing high levels of host (>10^{9}/ml; see figure). This phage has been chosen because it yields very good quantities of high quality DNA by the Wizard® Kit
protocol.



Starting at Section IV of the manual:

Removal of Lambda Phage Coat

Thoroughly mix the Wizard® Lambda DNA Purification Resin before removing
an aliquot. If crystals or aggregates are present, dissolve by warming the resin to 25–37°C for 10 minutes. The resin itself is insoluble. Cool to 30°C before use.

1) If you have not already done so, add the Nuclease Resuspension Solution provided with the system to the tube containing the lyophilized Nuclease Mixture. Re-suspend by pipetting gently. DO NOT VORTEX. Add 40µl of resuspended Nuclease Mixture to 10ml of lysate Incubate at 37°C for 15 minutes.
2) Add 4 ml of the provided Phage Precipitant, mix gently and place on ice for 30 minutes.
3) Centrifuge at 10,000 g for 10 minutes.
4) Carefully decant the supernatant. Re-suspend the pellet in 500 µl of Phage Buffer by pipetting gently. Pipette the Phage Buffer along the sides of the centrifuge tube several times to ensure complete resuspension of the phage pellet.
5) Transfer the resuspended phage to a 1.5ml microcentrifuge tube.
6) Centrifuge at 10,000 × g in a microcentrifuge for 10 seconds to remove any insoluble particles. Draw up the supernatant, being careful not to disturb the pellet, and transfer to a new microcentrifuge tube.
7) Add 1ml of thoroughly mixed Purification Resin to the supernatant and mix by inverting the tube.

Lambda DNA Purification Using a Vacuum Manifold

1) For each lambda lysate preparation, prepare one Wizard® Minicolumn as follows. Attach the Syringe Barrel to the Luer-Lok® extension of each Minicolumn. Insert the tip of the Minicolumn/Syringe Barrel assembly into the vacuum manifold.
2) Pipette the resin/lysate mix into the Syringe Barrel. Apply a vacuum to draw the resin/lysate mix into the Minicolumn. Break the vacuum to the Minicolumn at the vacuum source.
3) To wash the column, add 2ml of 80% isopropanol to the Syringe Barrel, and reapply the vacuum to draw the solution through the Minicolumn.
4) Dry the resin by continuing to draw a vacuum for 30 seconds after the solution has been pulled through the column. **Do not dry the resin for more than 30 seconds.** Remove the Syringe Barrel and transfer the Minicolumn to a 1.5ml microcentrifuge tube.

   Centrifuge the Minicolumn at 10,000 × g in a microcentrifuge for 2 minutes to remove any residual isopropanol.
5) Transfer the Minicolumn to a new microcentrifuge tube. Apply 100µl of water or TE buffer **preheated to 80°C**. Immediately centrifuge the Minicolumn at 10,000 g in a microcentrifuge for 20 seconds to elute the DNA.
6) Remove and discard the Minicolumn. The purified lambda DNA may be stored in the microcentrifuge tube at 4°C or –20°C. Resin fines may come through into the final eluate and appear as a fine white pellet in the eluate tube. The fines may affect subsequent applications or

spectrophotometric readings. To avoid these problems, centrifuge the eluate at top speed in a microcentrifuge for 1–2 minutes. Transfer the eluate to a tube, avoiding the resin pellet.

Relevant publications

The following articles describe nucleic acid extraction for marine viruses and their hosts. Adapting the protocols described in these papers is a good starting point for any work on marine viruses:

Schroeder, D.C., Oke, J., Malin, G. & Wilson, W.H. Coccolithovirus (Phycodnaviridae): Characterisation of a new large dsDNA algal virus that infects Emiliania huxleyi. Archives of Virology 147, 1685-1698 (2002).

Wilson, W.H. *et al*. Complete Genome Sequence and Lytic Phase Transcription Profile of a Coccolithovirus. Science 309, 1090-1092 (2005).

Allen M.J., Forster T., Schroeder D.C., Hall M., Roy D., Ghazal P., Wilson, W.H. 2006. Locus-specific gene expression pattern suggests a unique propagation strategy for a giant algal virus. Journal of Virology 80:15.

Jiang, S.C., C.A. Kellogg, and J.H. Paul. 1998. Characterization of marine temperate phage-host systems isolated from Mamala Bay, Oahu, Hawaii. Appl. Environ. Microbiol. 64: 535-542

Williamson, S., M. R. McLaughlin, and J.H. Paul. 2001. Interaction of a marine virus with its host: Lysogeny or pseudolysogeny? Appl. Environ. Microbiol. 67:1682-1688

Virus productivity

Markus. G. Weinbauer & Susan Kimmance

INTRODUCTION

The virus dilution approaches to estimate viral production has been initially published by Wilhelm et al. (2002). The rationale behind is simple. Viruses are ‘diluted, i.e. their abundance is reduced in order to stop or strongly reduce new infection. An increase in abundance over time is that due to already infected cells and this increase is viral production. In the initial protocol, a water sample was placed in a filtration unit equipped with a 0.2 µm filter. While the water sample was filtered, bacteria were kept in suspension using a syringe and the water volume was kept constant using virus-free water made by using a 30,000 MWCO cartridge. In a modification of the method, which is presented in detail below, a bacterial concentrate was made using 0.2-µm cartridges and this concentrate was diluted with virus-free water (Weinbauer et al. 2002). Recently a diafiltration approach was applied, using a tangential flow 0.2 -µm filter cartridge, in which the initial volume is kept constant using virus-free water.

100,00 MWCO cartridges have been used to remove viruses and in a variety of samples from coastal, offshore and deep waters, no viruses were found in the permeate (personal observation). Since in some of these approaches, viruses are not diluted but reduced, the term virus reduction approach has been used (McDaniel et al. 2002). In addition true dilution approaches have been sued to assess grazing and recently they have been applied to estimate virus-induced mortality of phytoplankton (Evans et al. 2003). To avoid confusion, the term virus reduction approach is used in the following.

Other methods to estimate virus production have been used as well and recently a comparison of some of these methods (including the virus reduction approach) has been made (Helton et al. 2005). More details on pros and cons of methods to estimate viral production can be found in Noble & Steward (Noble & Steward 2001) and Winget et al. (2005).

MATERIALS

Materials
Vivaflow 200 units, 0.2 -µm and 100,000 MWCO (includes tubing, fittings and screws); VIVASCIENCES

Peristaltic pump (we have very good experience with Watson & Marlowe)

Sampling bottles (2 - 5 liters)
Container for waste and cleaning solution and for the permeate (virus-free water)

50ml plastic tubes

Solutions
mQ
Ethanol 10%
Cleaning solution: 40g NaOH, 16ml commercial bleach (or 0.5M NaOCl) in 2L mQ

PROCEDURE

1. *Preparation of the system*

-remove the screws from the cartridge and connect tubes
-flush unit with 500ml mQ to remove the storage solution (ethanol 10%). When rinsing the system do not re-circulate.

2. *Concentration of bacteria (0.2 -µm cartridge)*

-place the feed tubing in the sample for the retentate (bacterial concentrate)
-flush the system with 50ml of sample
-place the feed and retentate tubing in the sample bottle
-start concentration at ≤2bar
-keep 50ml permeate
-when the volume of the sample (retentate) is around 50ml, transfer the sample in a plastic tube
-continue concentration procedure and when 20ml remain switch the system off
-put the retentate tubing in the plastic tube with the permeate
-reverse flow and fill plastic tube to 40ml (this increases the recover efficiency)

3. *Virus-free water (100,000 MWCO)*

- virus-free water is produced using 0.2-µm filtered water (see above)

4. *Virus production assays*

-Dilute bacterial concentrate (equivalent to 150 ml) with virus-free water to 150 ml and distribute in three 50 ml plastic tubes
-When the recovery efficiency is known (often 50 – 70% using the suggested volumes) bacterial abundance can be adjusted to in situ conditions
-take samples for counting bacterial and viral abundance in 3 hrs intervals
-typically a 12 h incubation is sufficient but this has to be tested for environments

5. *Cleaning procedure (ca. 1 hour)*

- flush the system with 50ml of filtrate
- flush the system with 200ml mQ
- place the feed tube in a container with 250ml cleaning solution
- flush system with 50ml cleaning solution
- place all 3 tubes in the container with cleaning solution and re-circulate for 30-40 minutes
- place all 3 tubes in a container with 250ml mQ and re-circulates for 5-10 minutes
- flush the system with 500ml mQ
- fill the system with a solution of ethanol 10% using the pump
- tight the tubes with the screws (alternatively, leave tubes on system and keep ethanol within it and the tubes by using clamps)
-store the system at 4°C

Tips:
- tubing tend to get disconnected from the cartridge. Fix tubing with tape.
- label tubing with tape (e.g. with different colors) to avoid confusion of tubing

COMMENTS

Virus production is estimated by least square regression analysis or by the increase in viral abundance with time. More than one maximum in viral abundance has been observed occasionally and in this case values have to be added (Winter et al. 2004).

When a peristaltic pump with several pump heads is used, several samples can be run in parallel. This is e.g. useful when a depth profile or experimental replicates are investigated.

In all virus reduction approaches, treatments result in a reduction of bacterial abundance. In the presented approach, an adjustment to in situ abundance can be made, either due to known recovery efficiency or by counting bacterial abundance in the bacterial concentrate.

ESTIMATING VIRUS-MEDIATED MORTALITY FROM VIRAL PRODUCTION

Dividing virus production by an assumed or measured burst size (Parada et al. 2006) yields the lysis rate of bacteria. If bacterial production data are available, virus-mediated mortality can be estimated by dividing the cell lysis rate by bacterial production rate (in cells per volume per time). If bacterial production data are not available for T0 but only for in situ, cell lysis rates have to be corrected for the fraction of bacteria lost during the treatment. If bacterial abundance data are available, virus-mediated mortality can be expressed as % loss per day.

The number of viruses produced can be divided by a burst size to estimate the frequency (better: percentage) of infected cells (FIC). FIC can be converted to virus-mediated mortality by using models (Proctor et al. 1993, Binder 1999).

REFERENCES

Binder B (1999) Reconsidering the relationship between virally induced bacterial mortality and frequency of infected cells. Aquat. Microb. Ecol. 18:207-215

Evans C, Archer S, Jacquet S, Wilson W (2003) Direct estimates of the contribution of viral lysis and microzooplankton grazing to the decline of a *Micromonas* spp. population. Aquat Microb Ecol 30:207-219

Helton R, Cottrell M, Kirchman D, Wommack K (2005) Evaluation of incubation-based methods for estimating virioplankton production in estuaries. Aquat Microb Ecol 41:209-219

McDaniel L, Houchin L, Williamson S, Paul J (2002) Lysogeny in marine *Synechococcus*. Nature 415:496

Noble R, Steward G (2001) Estimating viral proliferation in aquatic samples. In: Paul J (ed) Methods in Microbiology, Vol 30. Academic Press, San Diego, p 67-84

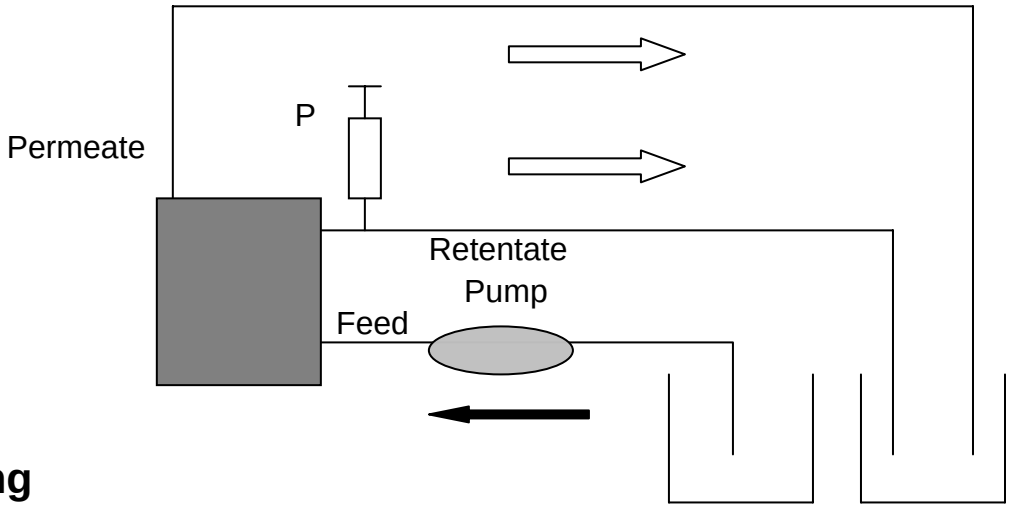
Parada V, Herndl GJ, Weinbauer MG (2006) Viral burst size of heterotrophic prokaryotes in aquatic systems. J. Mar Biol Assoc UK 86:613-621

Proctor LM, Okubo A, Fuhrman JA (1993) Calibrating estimates of phage-induced mortality in marine bacteria: ultrastructural studies of marine bacteriophage development from one-step growth experiments. Microb. Ecol. 25:161-182

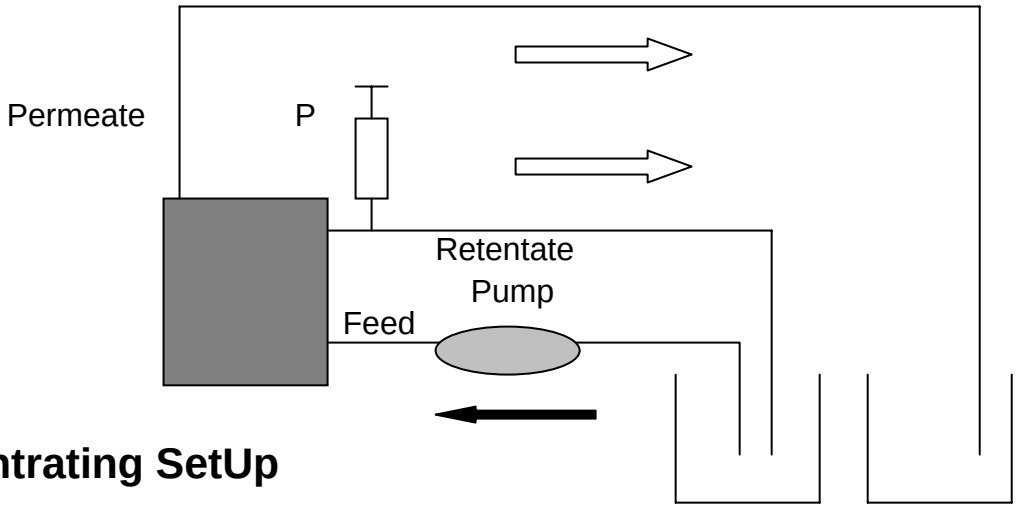
Weinbauer M, Winter C, Höfle M (2002) Reconsidering transmission electron microscopy based estimates of viral infection of bacterioplankton using conversion factors derived from natural communities. Aquat. Microb Ecol 27:103-110

Wilhelm S, Brigden S, Suttle C (2002) A dilution technique for the direct measurement of viral production: a comparison in stratified and tidally mixed coastal waters. Microb. Ecol. 43:168-173

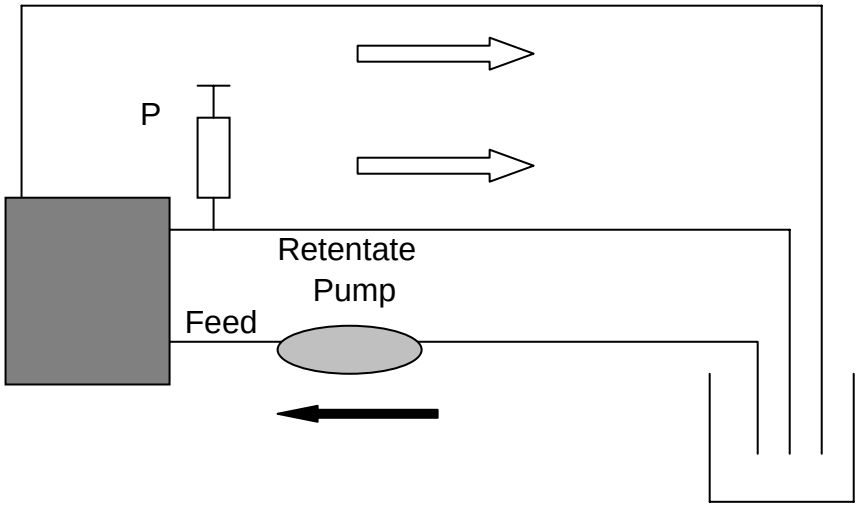
Winget D, Williamson K, Helton R, Wommack K (2005) Tangential flow dilafiltration: an improved technique for estimation of virioplankton production. Aquat Microb Ecol 41:207-216
Winter C, Herndl G, Weinbauer MG (2004) Diel cycles in viral infection of bacterioplankton in the North Sea. Aquat Microb Ecol



Flushing



Concentrating SetUp

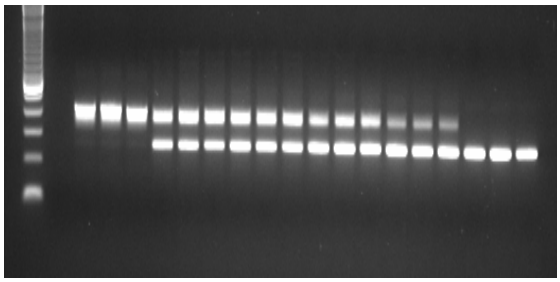


Quantitative PCR

Expert: Nicky Burbidge, Techne. Demonstrator: Karen Weynberg

PCR and Quantitative PCR

The Polymerase Chain Reaction (PCR) amplifies small amounts of specific regions of DNA so that there is sufficient DNA for analysis. Traditionally the DNA molecules are then detected using agarose gel electrophoresis, staining with ethidium bromide and visualisation under UV illumination. This visualisation strategy has some limitations such as a narrow dynamic range, size-based discrimination and only being semi-quantitative.



Specific amplification of 2 PCR products.

The first lane, M contains a molecular weight marker.

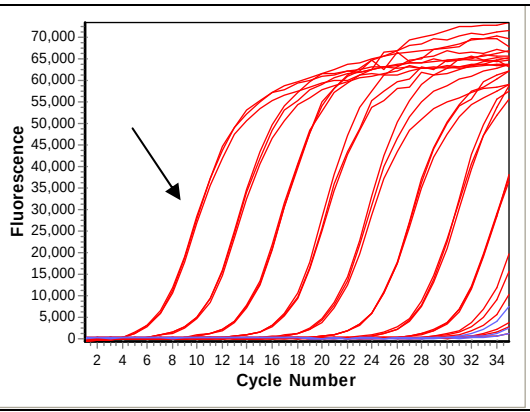
Quantitative PCR or QPCR is one of the most powerful molecular techniques commonly available today and can remove all of these limitations from the experiment. When using a quantification method it allows the accurate determination of the starting number of DNA molecules in each sample.

What are the advantages of QPCR?

The main advantages of the technique are the real-time monitoring of the PCR, faster analysis of the results and elimination of the possibility of cross-contamination of samples as there is no requirement for gel electrophoresis. Increased sensitivity and dynamic range, 2-fold discrimination in concentration and specificity are also important advantages. It is possible with the current instrumentation to detect 4 or 5 PCR products in the same sample at the same time thus saving time and cutting costs.

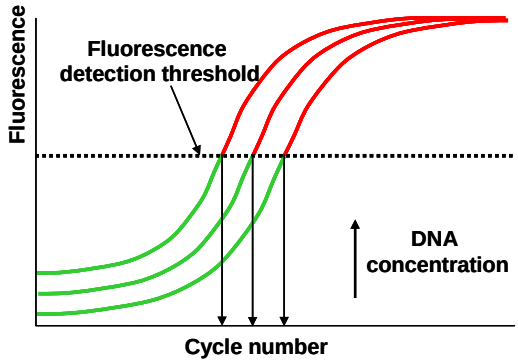
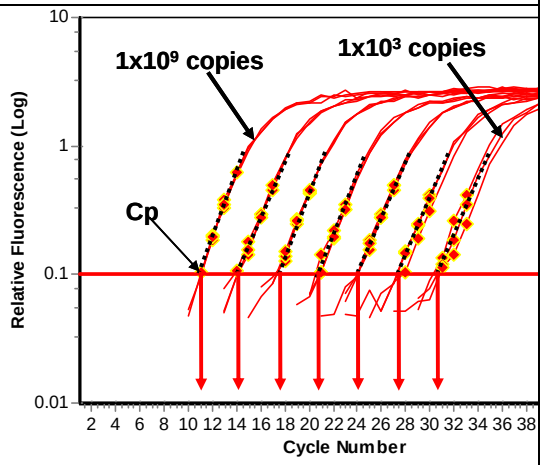
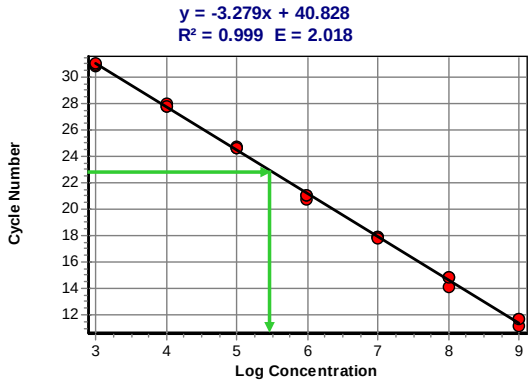
How does Quantitative PCR work?

The QPCR assay includes a fluorescent reporter; either a fluorescently labelled probe or a DNA binding dye which is measured during every cycle of the PCR. The increase in fluorescence is directly related to the number of DNA molecules present, with the samples containing a higher initial copy number (Cn) of the specific DNA molecule amplifying first.



QPCR data showing a 10-fold standard curve

The arrow indicates the sample with the highest Copy Number (Cn)

The samples are compared to each other by assessing when each amplification curve crosses a defined threshold which is set in the exponential phase of the reaction.	
The point at which the amplification curve crosses the threshold is called the Crossing Point (Cp) or the Cycle Threshold (Ct).	
The use of standards of a known DNA concentration allows the concentrations of unknowns to be calculated. The Cp of the standards is plotted against the log of the initial concentration as shown. Unknown samples are then compared to the standard curve.	
During an efficient PCR each cycle results in an increase in the DNA molecules by a factor of two. A difference of one Cp between two samples therefore represents a 2-fold difference in expression.	

QPCR Chemistries

The two most common QPCR chemistries are SYBR® Green I and TaqMan™ hydrolysis probes.

SYBR® Green I

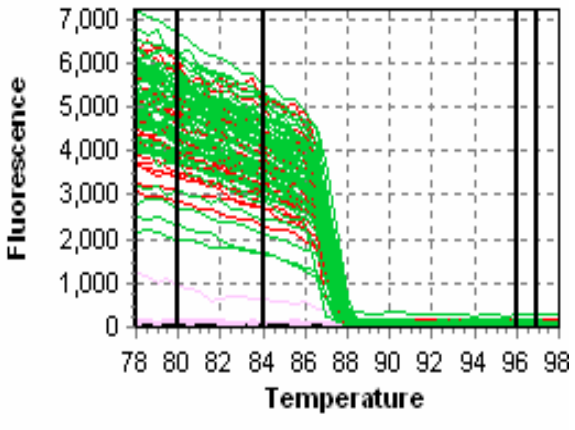
SYBR® Green I is a universal chemistry and only requires a set of gene-specific primers, thus it is easy to design and relatively low cost to perform. This chemistry uses the same mechanism as ethidium bromide, in that it binds in the minor grove of any double-stranded DNA and fluoresces. If the fluorescence is measured at the end of the extension step of the PCR then the DNA is double-stranded, the SYBR® Green I is bound and there is a fluorescence signal relative to the number of double-stranded

DNA molecules present. Due to the dye binding to all double-stranded products it is not usually possible to analyse more than one gene at a time.

One useful feature of SYBR® Green I is that it is possible to perform a dissociation curve. The two strands of DNA will separate or "melt" at a temperature which is specific to individual amplicons, depending on the length of the product and the actual sequence of bases. The point at which the DNA strands separate results in a sharp decrease in the fluorescence signal.

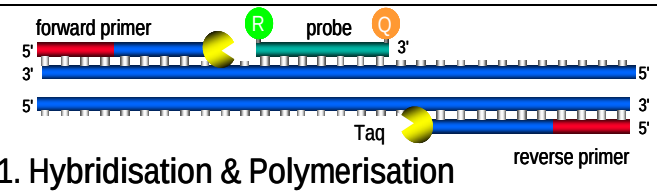
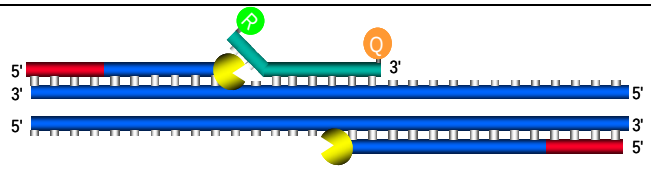
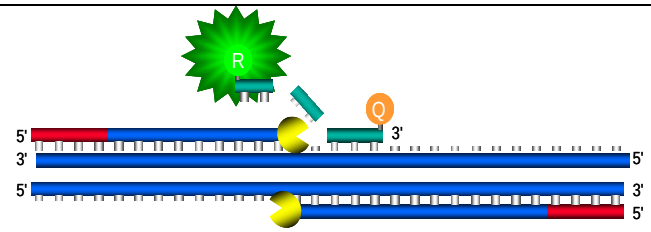
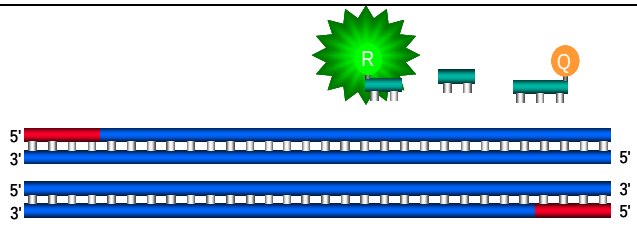
A piece of DNA with more C and G bases will melt at a higher temperature than an AT rich sequence, due to the higher number of hydrogen bonds between the two strands of DNA.

The melting temperature can then be used to confirm that the product amplified is the correct. Non-specific products ands primer-dimers will melt at a much lower temperature.



TaqMan™

The TaqMan™ assay uses an extra labelled primer, a probe, which is specific to the gene and binds between the forward and reverse primers. This makes the chemistry specific to the gene of interest and allows more than one set of primers and probe, if labelled with different fluorescent dyes to be used at the same time to detect multiple genes. This is commonly called multiplexing. The TaqMan™ probe is also called a dual-labelled probe as the probe has 2 molecules attached to it; the reporter dye is attached to the 5' end and a quencher at the other end. The reporter dye is excited by the instrument and when the two molecules are close together the reporter dye transfers its energy via FRET to the quencher. The quencher then releases the energy as light at a different, longer wavelength or heat. As the instrument is only detecting any light emitted from the reporter dye none is detected from the quencher. The steps of the assay are shown below.

 1. Hybridisation & Polymerisation	The first step in the TaqMan assay is the hybridisation of the primers and labelled probe to the target DNA. The Taq DNA polymerase extends the primers in the 5' to 3' direction.
 2. Strand Displacement	The Taq then displaces the labelled probe from the template DNA. The probe cannot act as a primer due to the 3' end being blocked.
 3. Cleavage R = Reporter Q = Quencher	One of the characteristics of Taq DNA polymerase is that it has 5' to 3' exonuclease activity, which cleaves the displaced probe. This releases the reporter dye and separates it from the quencher, leading to an irreversible increase in fluorescence. The amount of fluorescence is related to the number of molecules of DNA produced in the PCR.
 4. Polymerisation Completed	The Taq continues copying the DNA template.

The most common application is in gene expression where a duplex is performed, one of the genes is the experimental gene and the other is a control or house-keeping gene.

What can QPCR be used for?

There are many applications including:

- Gene expression: Presence (or absence) of specific genes in cell or tissue type
- Detection and quantification of pathogen numbers: Viruses, bacteria and fungi
- Genetically modified organisms testing (GMO)
- Single cell QPCR
- SNP detection
- Mutation detection between variants

For extensive information relating to all aspects of real-time PCR see the following website:
http://www.gene-quantification.info/

Quantica Real-Time PCR SYBR® Green Assay

Introduction

The primers used in this assay are very specific and have been designed to only amplify the gene for the major capsid protein from EhV. This protocol enables the amplification and subsequent detection of a 2-fold dilution series of standards of a known concentration using SYBR® Green. These standards can then be used to accurately determine the amount of DNA from EhV in the unknown samples.

The DNA standards have concentrations of 4ng/µl, 2ng/µl, 1ng/µl, 0.5ng/µl, 0.25ng/µl and 0.125ng/µl. A blank or No Template Control (NTC) is also required.

200µl	2x Polymerase mix
40µl	10x Primer mix
10µl	50x SYBR® Green
200µl	Nuclease-free water

15µl Of each standard and the unknown sample

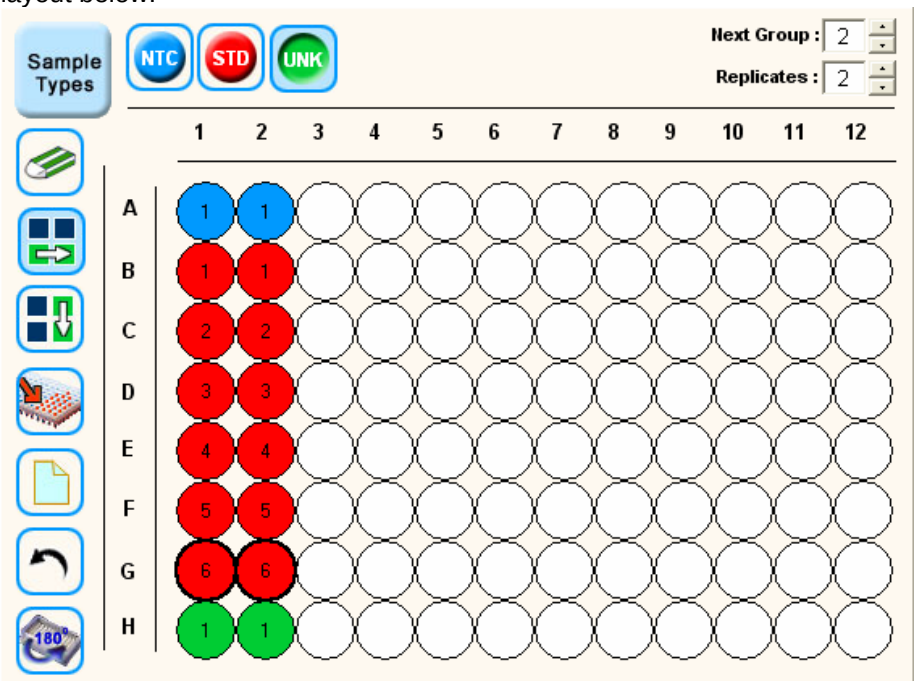
Preparation of the samples

1. Prepare the master mix as shown in the table below in a 1.5 ml microfuge tube. This is sufficient for 18 x 20µl reactions.

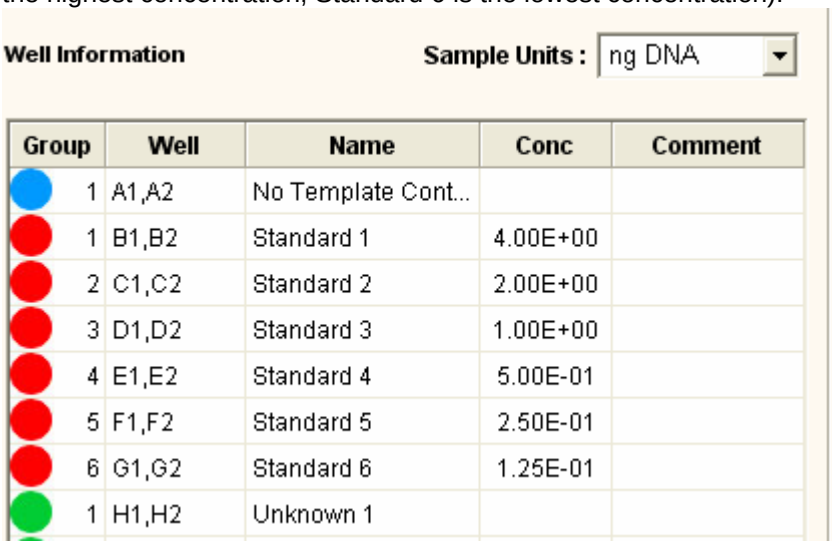
	Volume per reaction	18 x 20µl reactions
2x Polymerase mix	10µl	180µl
10x Primer mix	2µl	36µl
50x SYBR® Green solution	0.4µl	7.2µl
Nuclease-free water	2.6µl	46.8µl

2. Mix thoroughly by flicking the tube then centrifuge briefly.

3. Aliquot 15µl of mix into each of the wells of the PCR plate as shown in the plate layout below.



4. Add 5µl of the standards, unknown or water to the appropriate wells to give the **final** concentrations as detailed in the well information table below (Standard 1 is the highest concentration; Standard 6 is the lowest concentration).



Well Information **Sample Units :** ng DNA

Group	Well	Name	Conc	Comment
1	A1,A2	No Template Cont...		
1	B1,B2	Standard 1	4.00E+00	
2	C1,C2	Standard 2	2.00E+00	
3	D1,D2	Standard 3	1.00E+00	
4	E1,E2	Standard 4	5.00E-01	
5	F1,F2	Standard 5	2.50E-01	
6	G1,G2	Standard 6	1.25E-01	
1	H1,H2	Unknown 1		

5. Seal the plate using the optically clear flat caps and place in the real-time thermal cycler.

6. Perform the following thermal cycling program for 30 cycles:

Stage	Number of Cycles	Temperature	Time	Fluorescence Read
Enzyme Activation	1	95°C	10 min	None
Amplification	30	95°C	10 sec	None
		51°C	20 sec	None
		72°C	20 sec	**FC02:** 10% ND, 150ms Integration time.
Dissociation Curve	79	51-90°C in 0.5°C increments	10 sec	**FC02:** 10% ND, 150ms Integration time.

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Notes on licences:

1) Purchase of this product includes limited rights to use the supplied amount of SYBR® Green I Stain patented by Molecular Probes Inc. SYBR® Green I is a registered trademark of Molecular Probes Inc.

2) Purchase of this product does not convey a licence to perform any patented process.

Friday 28th July

Time	Activity			Location
0900 - 1000	Case study: John Paul **Prophages: Dangerous Molecular Time Bombs or the Key to Bacterial Survival in the Oceans?**			Resource centre
1000 – 1030	Methods briefings			Resource centre
1030 - 1100	COFFEE			Mess room
1100	Students split into 3 groups (Red, Green & Blue) **EFM:** Epiflourescence Microscopy for virus counting (Curtis Suttle & Steve Ripley) **PFGE:** Pulsed Field Gel Electrophoresis (Eric Wommack & Jayme Lohr) **DGGE:** Denaturing Gradient Gel electrophoresis (Ruth-Anne Sandaa, Joaquin Martinez Martinez & Declan Schroeder)			MBA
	EFM Room 82	**PFGE** Room 5	**DGGE** Room 76	
1100 – 1230	Red	Green	Blue	
1230 - 1330	LUNCH			Mess room
1500 - 1530	Green	Blue	Red	
1530 - 1600	COFFEE			Mess room
1530-1700	Blue	Red	Green	
1700 - 1900	**GET YER KILTS ON!**			
1900 -	BURNS NIGHT			Mess room

Notes:

Friday 28th July

Enumeration of Viruses by Epifluorescence Microscopy

Experts: Curtis Suttle & Amy Chan. Demonstrator: Steven Ripley

Course notes prepared by Curtis A Suttle and Amy M Chan, based on Ortmann and Suttle (in press)

Introduction

Epifluorescence microscopy has become a routine approach for estimating the abundance of viral particles in aquatic samples. The method is accurate and precise and can be done using optical equipment that is standard equipment in many marine microbiological laboratories. The basic approach is to collect the viruses onto a membrane filter and stain with a fluorescent dye. The first dye that was used to quantify viruses in marine samples by epifluorescence microscopy was DAPI (4'6-diamidino-2-phenylindole) (Suttle et al. 1990, Hara et al. 1991), which fluoresces light blue when bound to DNA and excited with UV. Unfortunately, the small amount of DNA in viruses and the relatively low quantum yield of DAPI meant that the particles were at the limit of detection in many epifluorescence microscopes.

The advent of dyes with a much higher quantum yield resulted in a method that was routinely accessible in laboratories and shipboard. The first of these dyes to be used was Yo-Pro (Hennes & Suttle 1995). The dye displays a very bright and stable fluorescence and estimates of viral abundance obtained using this method are more accurate and precise than typically obtained by epifluorescence microscopy. One downside of the method is that staining requires 48 h and aldehydes interfere with staining; hence, the method is not suitable for use with preserved samples. Subsequently, two other dyes, SYBR Green I (Nobel & Fuhrman 1998), and SYBR Gold (Chen et al. 2001) were introduced. The staining time for these dyes was only 15 minutes, and initially it was reported that they could be used on aldehyde-preserved samples. Consequently, these dyes were rapidly adopted by many investigators. In fact, estimates of viral abundance in aldehyde preserved samples decreases exponentially, so that within a matter of hours, the abundance of viruses is grossly underestimated (Wen et al. 2004). Consequently, viral abundance has been underestimated in many studies on natural samples (Suttle 2005). Moreover, the fluorescence of the SYBR stains (particularly SYBR Green) is much less stabile than for Yo-Pro, and needs to be chemically stabilized. The loss of viral abundance in fixed samples, and low fluorescence stability is probably the reason that some authors have concluded that viral abundance is underestimated in SYBR-Green stained samples (Bettarel et al. 2000). In fact, if the samples are carefully prepared using appropriate protocols, either Yo-Pro or SYBR stains can be used to obtain accurate estimates of viral abundance in aquatic samples (Wen et al. 2004).

In the Suttle laboratory, both Yo-Pro and SYBR are used to stain viruses in laboratory and field samples. In our hands, Yo-Pro consistently produces brightly-stained viruses, stable fluorescence and very reproducible estimates of viral abundance. Consequently, where sample is limited or it is not possible to check the quality of the slides, we typically use the Yo-Pro protocol (Hennes & Suttle 1995). When counts need to be done quickly and the volume of sample is not limiting, SYBR staining is used (Noble & Fuhrman 1998) as outlined in Wen et al (2004). Because of the limited time available to us this week, we will use SYBR Green in today's laboratory exercise.

Materials

Equipment

1. Epifluorescence Microscope (excitation 497 nm, emission 520 nm; eg wide blue filter set) equipped with 100X objective
2. Filtration unit, with filter support to hold 25 mm diameter filters, receiver flask and tubing
3. Vacuum pump [vacuum between 5 and 7 mm Hg]
4. Pipettors with volume ranges, from 0.5-1000 uL
5. Tally counter
6. filter forceps, blunt ended, unserrated tips
7. squirt bottle [filled with mQ water]
8. vortex mixer

Disposables

1. 25 mm dia filters 0.45 um pore-size nitrocellulose (eg Millipore HAWP 02500)
   0.02 um pore-size Al_2O_3 (Whatman Anodisc, cat no. 6809-6002)
2. Microfuge tubes, for dilution of antifade and samples
3. Staining dishes: 100 mm dia. plastic petri dish
4. Microscope slides
5. Glass cover slips: 25 mm x 25 mm, No. 1 thickness
6. Low or non-fluorescent immersion oil (Cargylle DF or FF grade)
7. Pipette tips: volume from 0.5 uL to 1000 uL
8. Kimwipes or qualitative filter paper (90 mm dia)

Reagents

1. SYBR nucleic acid stain, 10,000X in DMSO (Invitrogen). Store in original packaging at -20ºC, dessicated and protected from light. Use gloves and protect skin.
2. Glycerol (aka glycerine)
3. 1x PBS (Phosphate buffered saline: 4.3 mM Na_2HPO_4, 137mMNaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, pH 7.4)
4. *p*-Phenylenediamine dihydrochloride, (eg Sigma cat no. P1519) - Store tightly capped and protect from air and light. Use gloves and protect skin.

5. 1x TE pH 8.0: 10mM Tris pH 8.0, 1mM EDTA

Methods

All steps involving the stain should be done in very low light. If possible, use red or indirect light in a dark room for slide preparation. Bright lights will cause bleaching of the stain and reduce the fluorescence of the virus particles.

Preparation of Reagents

For preparation of reagents use 0.02-um filtered deionized water. This is very important in the dilution of the stain and the samples to prevent the introduction of virus particles.

Stain: To make a stock solution, dilute the SYBR stain 1:10 with 0.02-um filtered deionized water. Aliquot small volumes into plastic microfuge tubes and store at –20 ºC. The stain is stable for about 1 month when prepared this way. SYBR tends to bind to plastics and glass, with the lowest binding to polypropylene. There is a decrease in the stain concentration over time via adsorption to the plastic. For long term storage, it is recommended that the stain is diluted and stored in DMSO as the original concentrated stock.

Antifade: Prepare a solution of 50:50 glycerol:PBS, and vortex to mix. Prepare 10 % stock solutions of *p*-phenylenediamine and store at -20ºC in small volumes (<50 uL) to minimize freeze/thaw cycles. When *p*-phenylenediamine oxidizes it turns tea-coloured or darker, and should be discarded. Immediately before making the slides prepare ~50 uL per slide of the working antifade solution (i.e. 0.1 % solution of *p*-phenylenediamine in 50:50 glycerol:PBS).

Preparation of Sample

1. The sample, especially those from cultures, should be diluted to about 10^7 mL^{-1} prior to collecting the viruses on the filter. The abundance of viruses should be low enough so that individual particles can be discriminated, but not so low that there are < ~10 viruses per field. It may be useful to prepare several dilutions of a sample with an unknown virus concentration to determine the best volume of sample to filter. It is best to dilute the sample to 800-1000 μL for filtration, as smaller volumes may not result in even filtering of the sample. An assumption of the method is that the viruses are evenly distributed on the filter. Dilution of the sample should be done with 0.02 μm filtered solutions. It is important that the solution used for dilution does not contain viruses. Tests may be required to determine the appropriate solution to use. If sampling from a culture, filtered media may be the best diluent. Marine samples should be diluted with 0.02 μm filtered seawater to maintain the salinity and prevent bursting of cells and viruses, while lake samples can be diluted with filtered lake water.

2. Samples to be stained with SYBR should be fixed with glutaraldehyde (0.5% final concentration) for up to 30 min at 4°C prior to preparing slides. For some samples this may improve the fluorescence of the particles and make counting easier. Storage of viruses fixed in aldehydes at 4°C is not recommended due to the loss of viruses during storage (Xenopoulos & Bird 1997, Danovaro et al. 2001, Wen et al. 2004). If fixation and storage of samples is necessary prior to the preparation of slides, the best method is to fix the samples in 0.5% EM grade glutaraldehyde for 15 to 30 min at 4ºC, then flash freeze in liquid N_2. The samples should then be stored at -86°C until the slides can be prepared (Wen et al 2004). When ready to prepare slides, the sample should be quickly thawed and processed to avoid loss of virus particles.

3. The abundance of viruses in sediment samples can also be determined using epifluorescence microscopy, but further steps are necessary to prepare the sample and remove particulates that may interfere with counting. Because viruses attached to soil or sediment particles can be impossible to see using epifluorescence microscopy, preparation of the sample requires that the viruses be dislodged from the particles. Danovaro et al. (2001) removed virus particles from sediments by mixing 0.5 mL of sample with 4.0 mL of 0.02-µm filtered distilled water and 1.0 mL pyrophosphate (10 mM final concentration), sonicating for 3 min and centrifuging at 800×g for 1 min. The supernatant is diluted and the slides prepared. For different types of sediments and soils, the amount of pyrophosphate and length of sonication may need to be tested and optimized. Surfactants such as Tween80 may also be added to enhance detachment of viruses from the sediment or soil; the concentration used and incubation time must be optimized.

Filtration and Staining of Sample

1. Prepare the working solution of the stain in a plastic container. For staining, plastic petri dishes work well. Up to 4 filters can be stained in one dish and the dishes can be reused. To prepare the working solution add 2 uL of stock solution of SYBR stain to 78 uL of 0.02 um filtered deionized water. One drop should be prepared for each filter. Yo-Pro slides have the benefit of having stable fluorescence without the need for an anti-fade solution. The down side is that the staining requires 48 h, although it can be reduced to a few min by using microwave irradiation (Xenopoulos & Bird 1997). The following modifications are necessary for staining with Yo-Pro-1 (Hennes & Suttle 1996). Prepare the sample and filter as above, but do not fix the sample as aldehydes interfere with staining. Place the filter on a 80µL drop of Yo-Pro-1 solution (50 mM Yo-Pro-1, 2 mM NaCN). Place a wet filter (9mm dia.) in the top of the petri dish to prevent drying and stain in the dark for 48 h. Place the filter back on the filtration unit and rinse twice with 0.02 µm filtered distilled water. Mount the filter on the slide using 100 % glycerol. The slide is then ready to be counted as above. Yo-Pro-1 excites at a wavelength of 491 nm and emits at 509 nm.
2. Prepare the filtration unit, connecting it to a vacuum source. The vacuum should be no higher than 7 mm Hg.

3. Set up filtration unit using 0.45 um, nitrocellulose filters as a backing filter. This filter can be reused several times, as long as it has no holes and remains flat. Make sure that this filter wets evenly; add a thin layer of mQ on top of the filter. Discard the filter if the color of the wet filter is not consistent throughout.
4. Apply a 0.02 um Anodisc filter over the wet backing filter. Add a thin layer of deionized water to the backing filter before placing the Anodisc filter on top of it. Turn on the vacuum to pull this water through. It is important that there is no air trapped between the filters or the sample will not be pulled through the Anodisc. When handling the Anodisc filter, hold it by the plastic ring around the membrane. Because of the characteristics of the Anodisc membranes, the filters will not bend, but crack. Check to make sure the membrane is not cracked before using the filter.
5. With the vacuum off, add the sample to the Anodisc. Without a filtration tower, water tension will allow 0.8 to 1.0 mL of sample to be placed on the filter. Make sure the entire volume is within the plastic ring, otherwise the sample will be pulled under the edge of the filter. Turn on the vacuum and filter the sample through the filter. If more than 1 mL needs to be filtered there are two options.
    a. If the total volume is 2 mL or less, add 1.0 mL of the sample and allow some to be filtered, adding more until all of the sample has been filtered. It is important that the filter does not dry out between additions of the sample.
    b. A filtration tower can be used for larger volumesThe tower must fit over the centre of the Anodisc and not cross over the plastic ring. Measure the interior diameter of the tower so the diameter of the filtration surface is known. Ensure that the fields counted are within the area of filtration.
6. Once the sample has been entirely filtered, carefully remove the filter while the vacuum is still on. When lifting the Anodisc, touch only the plastic ring and be careful not to bend and crack the membrane.
7. Place the filter, sample side up, onto a filter paper and allow the filter to air dry. The filter will appear opaque when it is dried.
8. Place the Anodisc, sample side up, on a drop of stain in a plastic petri dish. Allow the filter to stain for 15 min in the dark.
9. After 15 min, remove the Anodisc from the stain and place it on top of the 0.45 um backing filter (add a small amount of deionized water between the filters to ensure no air is trapped). Turn on the vacuum and pull any fluid back through the filter.
10. Remove the Anodisc from the filtration unit while the vacuum is still on. Place the Anodisc, sample side up, on a Kimwipe or filter paper and allow the filter to dry. When the filter is dry it will appear opaque.
11. When dry, mount the Anodisc onto a slide using the anti-fade solution. Place 10-15 uL of antifade on the slide and put the Anodisc on top. Add ~25 uL of antifade on top of the Anodisc and top with a coverslip. Gently press down on the coverslip to ensure that no bubbles are trapped between the filter and the coverslip. The amount of anti-fade solution used to mount the slide should be the minimum needed to fill the area between the filter and the coverslip. If the slides are going to be frozen before counting, more

anti-fade solution should be used to compensate for shrinking during freezing.

12. The slides can either be counted immediately or stored frozen at -20°C for as long as 4 months with no decrease in abundance of viruses or fluorescence. Multiple freezing and thawing of the slides should be avoided.

Determining Abundance

1. Using either DF or FF grade immersion oil count the viruses using the 100X objective. The particles will appear green (SYBR Green I or II) or yellowish (SYBR Gold) when excited with blue light.
2. Before starting to count the particles on the slide, check to ensure that the slide is "good." A good slide should have even staining with the sample evenly distributed across the entire filter. The sample should also be on a single plane with the fluorescent particles attached to the filter and not the coverslip or floating between the two.
3. There are two methods for counting the particles.
    a. The first method uses an ocular reticule with a grid divided into squares of known area. The number of particles within each field are then counted. The size of the field should be selected so that each contains ~10 particles. Particles should be counted that are within each area. For particles touching the edges of the grid select 2 sides (ie. left side and top) where particles are counted. Particles touching the other 2 sides (ie. right side and bottom) will not be counted.
    b. The second method uses a CCD camera to obtain an image of a field, which is then either counted by an individual or analysed with computer software (Chen et al. 2001). Using this method, the field is defined as the area of the image, so the sample needs to be at the correct concentration to allow ease of counting.
4. The number of fields counted, the size of the field, the total number of particles and the total volume of sample filtered needs to be recorded for each sample. The total area through which the sample was filtered must also be recorded. If no filter tower was used, this area is the total area of the filter (radius =12500 μm), otherwise the radius of the filter tower needs to be determined.
5. The following equation can be used to calculate the abundance of viruses (Suttle 1993):

$$N_v = P_t \div F_t \times A_t \div A_f \div V_t$$

N_v=viruses mL^{-1}	P_t=total number of viruses counted
F_t=total number of fields	A_t=total area filtered (μm^2)
A_f=area of each field (μm^2)	V_t=volume of sample filtered (mL)

6. The total number of particles counted will determine the size of the 95% confidence intervals that can be calculated for each sample. The 95% confidence intervals can be calculated using the following equations (Suttle 1993):

$$\text{Upper } 95\% = P_t + 1.96 \times \sqrt{(P_t + 1.5)} + 2.42$$
$$\text{Lower } 95\% = P_t - 1.96 \times \sqrt{(P_t + 0.5)} + 1.42$$

Because the confidence intervals are determined from the total number of particles counted, it is important to count sufficient particles to have the desired accuracy. As the number of particles counted increases, the rate at which the accuracy increases is reduced. The number of particles counted is therefore a trade-off between accuracy and effort. For instance if P_t=100, the 95 % CI is 82-122, which is equivalent to ±~20%. If the number of particles counted is increased to P_t=200, the 95 % confidence interval is 174-230, or ± ~14%. A further doubling of the particles counted, to P_t=400, only decreases the error to ± ~10%. In general, counting at least 200 particles in 20 fields is a recommended balance between accuracy and effort (13).

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Analysis of virioplankton communities by Pulsed Field Gel Electrophoresis

Expert: K. Eric Wommack. Demonstrator: Jayme Lohr,
Assoc. Professor University of Delaware

INTRODUCTION

Documentation of community diversity (richness of species) and composition (evenness of species) are fundamental elements of ecological research. For viral communities, a number of qualitative and quantitative approaches to cataloging diversity have been developed, yet all of these approaches an be broadly clustered into two groups. Genotypic approaches to characterizing viral diversity typically utilize polymorphisms within genetic sequence data as a qualitative proxy for the diversity of viral strains containing a specific marker gene (eg. DNA polymerase, terminase) (Chen, Suttle et al. 1996; Fuller, Wilson et al. 1998; Zhong, Chen et al. 2002). These approaches are analogous to characterizing bacterial species diversity based on sequence polymorphisms within the small subunit ribosomal RNA gene (16SrDNA) (Olson, D.J. Lane et al. 1986). However, unlike cellular life (eubacteria, archaea, and eukarya) there is no universal genetic marker capable of capturing all viral diversity. Moreover, all forms of polymeric nucleic acid, not just dsDNA, can serve as the genomic material of viruses. As such an important limitation of genotypic approaches to describing viral diversity is that only a subset of species within a given viral community can be assayed using a single marker gene.

An alternative to sensitive, but narrow, genotypic approaches, is to catalog viral diversity utilizing a phenotypic characteristic which is broadly shared by viruses. The most commonly used phenotype for description of viral diversity has been morphology of virus particles as assessed through transmission electron microscopy (TEM) (Wommack, Hill et al. 1992). Characterization of viral morphology through TEM has been critical to understanding the role of viral structural proteins in the infection process and, prior to the development of molecular genetics, was the only tenable means of describing viral diversity within environmental samples. Indeed, the discovery of high viral abundance within marine ecosystems came through direct TEM observation of virus particles within water samples. Morphological data has been a fundamental component in taxonomic classification of viruses (Ackermann 2001); however, the small range of viral morphological characteristics means that only low resolution descriptions of viral communities are possible based on TEM morphology of virus particles.

The ideal assessment tool for determining the richness and evenness of viral communities should target a characteristic which is shared by all viruses and varies broadly across the swath of viral diversity. One such characteristic is genome size. Among known dsDNA viruses genome size varies over 150 fold from ~8 kb for Papillomaviruses to ~1,200 kb for Mimivirus (Raoult, Audic et al. 2004). Variations in genome size for RNA and ssDNA viruses are not as dramatic and these genomes types are smaller, rarely exceeding 20 kb of total genomic nucleic acid. Thus, genome size is a phenotypic characteristic of sufficient variability and universality to characterize whole communities of dsDNA viruses. Although dsDNA viruses are only a subset of viruses, direct

counts of viruses within water samples, which are based on fluorescent stains which preferentially bind dsDNA, indicate that dsDNA viruses are extraordinarily abundant an aquatic environments (Weinbauer and Suttle 1997; Chen, Lu et al. 2001). Moreover, ~95% of all known bacteriophage contain linear dsDNA and aquatic viral communities are widely believed to be primarily composed of phage (Wommack and Colwell 2000). Thus, despite this limitation, the phenotype of genome size is capable of cataloging at high resolution, the composition and diversity of virioplankton.

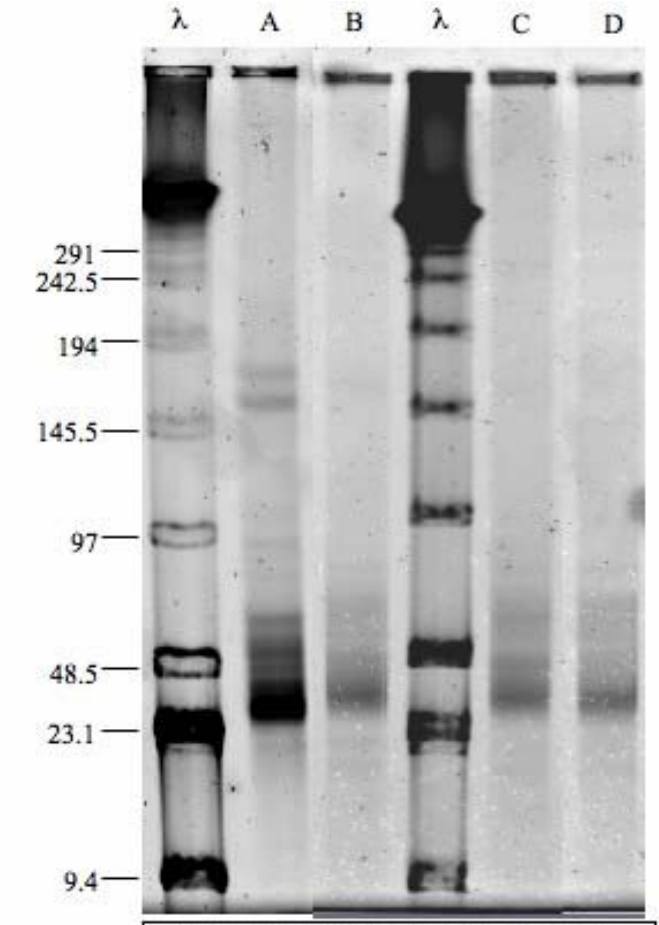

Fig. 1. Pulsed field gel of viral concentrates from the Chesapeake Bay run under conditions to optimize the separation of viral genomes between 10 and 300 kb in size. λ) molecular weight markers consisting of lambda concatamers and λ-hind III digest. A) Station 818, Mar. 2003; B) Station 818, Apr. 2003; C) Station 834, April, 2003; D) Station 744, April, 2003.

Currently, the best means for assessing the size of large, linear dsDNA is pulsed field gel electrophoresis (PFGE). Depending on electrophoretic conditions, PFGE is capable of resolving DNA fragments ranging from >1,000 to <10 kilobase pairs in length. This is made possible through alternating the orientation of the electrical field over a pulse interval of time. Because longer DNA molecules are slower to reorient to the changing electrical field, their net migration towards the cathode is slower. Detailed information on the theory and use of PFGE for molecular genetic analyses can be found in Birren and Lai (Birren and Lai 1993). In the case of viral communities, PFGE serves as a means to separate full length viral genomic DNA from a mixed community. The result is an electrophoretic profile, ie. fingerprint, of viral diversity and community composition according to the phenotype of genome size (Fig. 1). Each band on the PFGE gel represents a group of viral strains which share a common genome size. In practice, on a single gel 20 to 40 different bands can be resolved. This number can be increased by analyzing viral communities with different PFGE running conditions which effectively expands the range of detectible genome sizes (Fig. 2). While this number of characters is much smaller than the 100s of base pairs assayed in genotypic approaches, PFGE is capable of surveying a much greater range of viral diversity, i.e. all linear dsDNA viruses. An additional benefit of PFGE profiling of viral communities is that no amplification of viral genomic DNA is required. Thus,

the abundance of a given genome-size group of viruses within the total community can be determined based on the staining density of the band.

Initial use of PFGE in viral ecology was to profile viruses within sheep rumen (Klieve and Swain 1993). In subsequent application to virioplankton ecology, PFGE has revealed dynamic seasonal changes in virioplankton diversity over an annual cycle in the Chesapeake Bay (Wommack, Ravel et al. 1999) and over depth in near coastal oceanic waters (Hewson, Wingett et al. 2006). Virioplankton within several other aquatic environments including an alkaline lake have been surveyed using PFGE (Jiang, Steward et al. 2004). By and large viruses isolated from water samples have tended to give excellent results with PFGE profiling. In my experience, PFGE of viruses extracted from porous media samples, soil and sediment, have been more problematic for PFGE despite the 10 to 100 fold greater abundance of viruses in these environments.

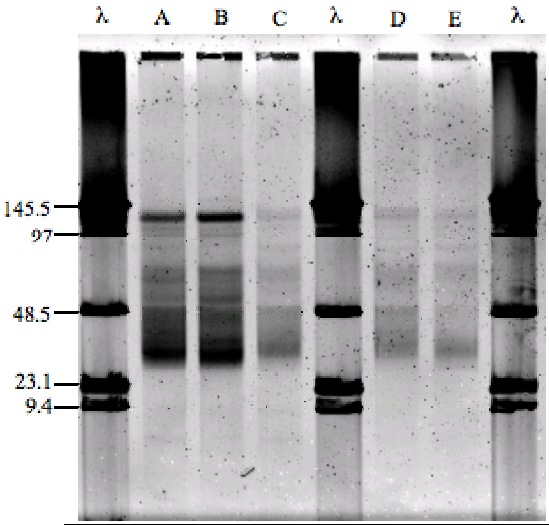

Fig. 2 Pulsed field gel of viral concentrates from the Chesapeake Bay run under conditions to optimize the separation of viral genomes between 20 and 100 kbin size. I) molecular weight markers consisting of lambda concatamers and l-hind III digest. A) Potomac river, Mar. 2003; B) Station 818, Mar. 2003; C) Station 818, Apr. 2003; D) Station 834, April, 2003; E) Station 744, April, 2003.

Obtaining a pure, high titer viral concentrate is the critical first step towards PFGE profiling of a virioplantkon community. Typically, this is done from large volumes of water (10s to 100s of liters) using a 30 to 100 kD molecular weight cut off filter and tangential flow filtration. Resultant viral concentrates are prepared for PFGE by suspending the viruses within an agarose plug or by further concentration and buffer exchange using spin ultrafiltration cartridges. It is also possible to process small volumes of seawater, <100 ml, using a series of spin columns and obtain enough virus particles for banding on a PFGE gel. Banding patterns can be compared using ordination approaches such as cluster and principal components analysis to describe changes in viral communities across environmental gradients (Wommack, Ravel et al. 1999; Riemann and Middelboe 2002). Southern blotting of PFGE gels followed by DNA hybridization analysis can be a useful means of detecting and quantifying the abundance of specific viruses within environmental samples (Wommack, Ravel et al. 1999). PFGE can also be used as a preparative technique to purify genomic DNA from specific viral strains within a mixed community.

LAB DEMONSTRATION

Introduction to pulsed field gel electrophoresis (PFGE)
Preparation of viral concentrates for PFGE
    Agarose plugs
    Spin Columns
Gel preparation
Gel loading
Set-up of CHEF-PFGE apparatus
Gel staining and imaging

PROTOCOLS

Sample preparation:

I)Post-Concentration of VCs for PFGE and other Techniques

A. Spin Filters (e.g., Centricon Plus 80, Centricon Plus 20)

1. Label one spin filter per sample to be concentrated. Be sure to label all parts of the spin filter (top, bottom, cap, recovery cap).
2. Pour each VC into the appropriately labeled spin filter. Fill it up, but leave enough room to put the cap on without overflowing.
3. Set up the table-top centrifuge (Eppendorf 5810 R) with the appropriate tube adapters (for Centricon 80s or Centricon 20s). Load filters.
4. Set run conditions: 4000 rpm (max for the centrifuge), 10 degrees C, acceleration rate 9, deceleration rate 9. Set time at 2 minutes for Centricon 20s, 5 minutes for Centricon 80s.
5. Start the run.
6. When run is complete, check the level of retentate. NEVER run the filters dry. Adjust the run time as needed to filter about 90% of the retentate volume. Drain the filtrate and refill the samples (retentate) as needed until the entire sample volume has been run through.
7. On the final run, (i.e., no more sample is waiting to be loaded into the spin filter) spin the tubes until no liquid can be seen freely floating above the filters. You should still be able to see liquid in the filter. A final wash can be performed with sterile buffer (e.g., SM buffer) if you prefer.
8. Insert the retentate recovery cap into the spin filter (see manufacturer's instructions for more details). Invert the filter/recovery cap assembly and load into the centrifuge (inverted).
9. Set run conditions: 1000X g, time = 4 minutes, all other conditions as listed above. Start run.
10. When run is complete, there should be a small volume of viral concentrate in the bottom of the recovery cap. Use a 200ul

(yellow) tip to carefully transfer to a sterile 2 ml eppendorf tube. Label the tube appropriately. Store at 4 degrees C until use.

B. Ultracentrifugation

1. Load SW 28 buckets with clean polyallomer tubes.
2. Pour VCs into tubes, keeping careful note of which VCs are in which tubes. Note also the weight of each tube and BALANCE opposite tubes to within +/- 1g.
3. Cap tubes and load into SW28 rotor. Be sure that tubes are firmly seated in the rotor.
4. Load rotor into centrifuge and run under the following conditions: 28 000 rpm, 4 degrees C, 12 hours, acceleration at max, deceleration at slow. Start run.
5. When run is complete and rotor has stopped, carefully remove tubes from rotor. Carefully pour or pipette off the supernatant, leaving about 5-10 ml of liquid in the bottom (a hazy film of phage may or may not be apparent on the tube bottoms). Pool the remaining 5-10 ml of phage concentrates in a 50 ml or 15 ml conical centrifuge tube. Make sure tubes are labeled.
6. Rinse each of the polyallomer tubes with 1 ml of SM, vortex briefly, and add to the pooled phage concentrate. Store at 4 degrees C until use.

II) Casting Viral Concentrates into Agarose Plugs

Solutions to prepare ahead of time:

1.5% In-Cert Agarose in SM buffer (make up in 50 ml tube)
250 mM EDTA + 1% SDS
TE buffer (10 mM Tris, I mM EDTA, pH 8.0)
Plug storage (20 mM Tris, 50 mM EDTA, pH 8.0)

A freshly prepared solution of Proteinase K (20 mg/ml) is also required.

1. Used plug molds are stored in 10% Liquinox. If you're using these molds, rinse the molds thoroughly with di water and dry before use. If you're using new plug molds, they're ready to go out of the bag. Break them apart before using and proceed to step 4.
2. Seal the bottoms of the molds with scotch tape. 2 molds can fit on one piece of tape (width).
3. Use a razor blade to carefully cut the tape between the plug molds, making sure that the bottoms of the wells stay sealed with tape.
4. Using small pieces of labeling tape, label each mold with the sample it is to receive.
5. Melt the 1.5% InCert agarose in the microwave. CAUTION: this type of agarose boils VERY quickly. Initially microwave 5 to 7 seconds. Then heat for 2-3 second intervals and swirl carefully between heatings until the agarose boils. Attend the microwave at ALL times during melting. After the agarose has completely melted, allow to cool briefly. Have

your viral concentrates ready; have the P 1000 set to 425 ul (or 500ul) and the P 200 set to 80 ul.

6. In a 2 ml Eppendorf tube, add 425 ul (or 500ul) * of viral concentrate and 425 ul (or 500ul) * of the melted 1.5% InCert agarose. Cap and vortex briefly.
7. Carefully dispense 80 ul of the mixture into each well of the appropriately labeled plug mold*.
8. Place the molds in the refrigerator (4 deg. C) for about 20 minutes to set.
9. Set up a tube for each mold: Pour about 2 ml of 250 mM EDTA + 1% SDS into a labeled 15 ml centrifuge tube. Add 100 ul of a freshly prepared Proteinase K solution (20 mg/ml).
10. Remove the tape from the bottom of the plug mold. Use a sterile disposable inoculation loop to push the lugs out of the mold and into the tube. The loop must be cut in half (along the axis of the handle) in order to fit through the wells of the plug mold.
11. Make sure all plugs are in the solution at the bottom of the tube. Incubate at room temp in the dark overnight.
12. The next day, carefully decant the Proteinase K/SDS solution, using a sterile disposable loop to hold back the plugs. Rinse the plugs 3 times, 30 minutes each, with 10 ml TE buffer. Use the loop to hold back the plugs while the liquid is carefully decanted each time.
13. Store plugs in 4-5 ml 20 mM, Tris 50 mM EDTA, pH 8.0 at 4 degrees C until use.

* Each well of the plug mold will hold 80 ul: 40 ul of virus suspension + 40 ul of 1.5% In-Cert agarose. A full mold has 10 wells and will require 10X these amounts, or 400 ul of virus suspension + 400 ul of In-Cert. Because of pipetting error and the viscosity of the agaose, it is necessary to add extra liquid to still end up with 10 full wells, hence 425 ul of virus suspension + 425 ul In-Cert. If you are making fewer than 10 plugs then scale back your volumes accordingly, but don't foget to allot extra for the pipetting error. (note from SB: I have found that if I use 500ul volumes that I will always have enough for 10 plugs and usually only have a small amount left over.)

III) Pulsed-field gel electrophoresis of viral concentrates

1. Make plugs – see plug-making protocol. Make sure there is at least 2.5 liters of 0.5x TBE already made.
2. Pour PFGE gel
    a. Make a 1% Biorad PFGE agarose gel in 0.5x TBE – for the large gel rig, the buffer volume is 200mL, so use 2g agarose (the small rig holds 100-150ml).
    b. Set-up gel rig - remember when putting in the comb to use microscope slides to ensure that the comb sits evenly along its entire length
    c. Pour in warm agarose and cool
    d. Cover with with 0.5x TBE (to prevent it from drying) and saran wrap (easiest in large pyrex dish). Place at 4^{o}C for at least 20min, preferably overnight.
3. Turn on the power for the Chef PFGE control unit.

4. Pour in 2.2L of cold 0.5x TBE to the PFGE rig (make sure it's been cleaned properly since the last use).
5. Turn on the pump (about 80/min is a good rate). Once the buffer is flowing, turn on the chiller.

**Important! - Do not run the chiller without running the pump, or the buffer may freeze in the lines. If this happens, turn off the pump and chiller and let the buffer thaw prior to re-starting the pump.

6. Set the temperature on the chiller and allow to equilibrate up while you load the gel.
7. Loading the gel:
    a. Cut the appropriate number of plugs (about 2mm wide) from the λ ladder – same number as ladder lanes on the gel.
    b. Prepare the Hind III digested λ ladder if needed – for 3 lanes, mix 40uL of ladder with 8uL of 6x loading dye and load 12uL in a lane.
    c. Remove the comb slowly and carefully. Load your ladder and samples into the gel wells.
    d. If you will be plugging your wells with agarose, load the liquid ladder before putting the gel in the rig, heat and melt 1.5% InCert agarose, and using a 1mL pipette, fill wells with agarose. Allow to cool and set,
    e. If you will not be plugging your wells, do not load the liquid ladder samples, skip step 7d and proceed to #8.
8. Set up gel in PFGE rig
    a. Make sure buffer temperature is at gel running temperature.
    b. Remove the gel from the pouring rig, and remove any extraneous gel from the bottom and edges with your hands.
    c. Place the gel in the PFGE rig. Make sure it is seated securely inside the frame all the way on the bottom of the rig.
    d. If loading liquid samples:
        i. turn off buffer chiller and pump briefly
        ii. load the samples
        iii. restart the pump and chiller.
9. Close the PFGE rig lid.
10. On the panel for the main PFGE unit, press the desired angle set-up, then input your run conditions as asked for.
11. When the run is done, press the number required to clear the program and stop the run
12. Turn off the chiller
13. Turn off the pump.
14. Spike the SYBR Gold bath with 25uL of stock SYBR gold, whisk with a pipette to evenly distribute the stain.
15. Lift out the gel on the plate and carefully slide the gel into the SYBR bath. Gently rock the bath and gel for 20-30 min, then remove the gel from the bath, place in de-stain, and scan on Typhoon imager.
16. Meanwhile, clean the the PFGE rig.
    a. Drain the buffer into the 10 L carboy labeled for "PFGE waste".
    b. Pour in 2L of DI water, turn the pump on, run for 5 minutes before draining
    c. Repeat for a total of 3 DI rinses.

d. After the third rinse, drain all lines, leave the lid open and the rig tilted up to continue to drain and dry.

17. Turn off the main power to the PFGE control unit.

Standard Run Conditions for virioplankton concentrates (25 to 300 kb genomes)

Run time:	22 hours
Initial Switch time:	1 second
Final Switch time:	15 seconds
Included Angle:	120 degrees
Voltage Gradient:	6.0 V/cm
Ramping factor:	linear (just hit enter)
Temperature:	14 degrees C
Initial Current:	Approx 140mA (record for each gel along with final current)

Optimized conditions for separating smaller genome fragments (25-100kb genomes)

Run time:	28 hours
Initial Switch time:	0.1 second
Final Switch time:	4 seconds
Included Angle:	120 degrees
Voltage Gradient:	6.0 V/cm
Ramping factor:	linear (just hit enter)
Temperature:	14 degrees C
Initial Current:	Approx 140mA (record for each gel along with final current)

ALTERNATIVE APPROACHES

As described in the introduction, PFGE, is currently the least selective means of profiling the diversity and composition of whole aquatic viral communities. Overall, the only significant methodological difference in the application of PFGE to profiling viral communities has been in the approach to loading viral concentrates on the PFGE gel. Most PFGE requires that DNA extraction occur within an agarose plug to prevent shearing. Several investigators have successfully demonstrated that DNA can be released from viral particles in solution, and that this extracted DNA can be directly loaded onto the PFGE gel. Steward (Steward 2001) presents an excellent protocol for solution-based preparation of viral DNA for PFGE. While the agarose plug method has the advantage of longer storage and enabling a single preparation for multiple get runs; the solution-based protocol is faster, i.e., does not require the overnight protease step, and can produce sharper banding.

EQUIPMENT AND CONSUMABLES SUPPLIERS

Tangential flow filtration and ultrafiltration spin columns

Millipore Corporation
(http://www.millipore.com/)

Clamped homogenous electrophoretic field (CHEF) – PFGE apparatus and consumables

Bio-Rad Laboratories
(http:// www.bio-rad.com)

PFGE specialty agarose and other consumables for electrophoresis

Cambrex Corporation
(http://www.cambrex.com/Content/bioscience/catnav.oid.901)

Invitrogen – Molecular Probes
(http://probes.invitrogen.com/)

Gel imaging light box for SYBR stains

Claire Chemical (Dark Reader Gel light box)
(http://www.clarechemical.com/)

Laser Gel Scanner

GE Healthcare (formerly Amersham Biosciences)
(http://www1.amershambiosciences.com/)

Mirai Bio (a division of Hitachi Software)
(http://www.miraibio.com/)

Gel analysis software

Scanalytics (a division of BD Biosciences)
(http://www.scanalytics.com/)

Applied Maths
(http://www.applied-maths.com/)

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I am greatly indebted to the following students and colleagues who have collectively refined these methods for PFGE profiling of viral communities: Jacques Ravel, Shellie Bench, Kurt Williamson, Danielle Winget, Shannon Williamson, and Matt Simon.

Denaturing Gradient Gel Electrophoresis

Expert: Ruth-Anne Sandaa.
Demonstrators: Joaquin Martinez Martinez, Declan Schroeder

DGGE scedule;

Total time; 90 min
Each group will be subdivided in a further three groups, each 3-4 students.

1. General information, principle of DGGE.
2. Casting a DGGE gel.
3. Loading and running a pre-poured DGGE gel.
4. Take down a pre-run DGGE gel and stain
5. Look at a pre-run and stained gel, demonstration of DGGE analysing program.

Denaturing gradient gel electrophoresis (DGGE)

1.0 Theory

Denaturing gradient gel electrophoresis (DGGE) fingerprinting, based on polymerase chain reaction in combination with DGGE, is a commonly used technique to analyse community composition and structure in microbial communities. The analysis gives information about the most dominant populations in the community, and combined with sequencing it is possible to gain phylogenetic information about these organisms. The method enables us to rapidly screen multiple samples and obtain valuable information about community shifts in time and space. The technique is also useful for detecting changes in the microbial community due to the effect of different treatments or environmental factors. The limitation of the method mainly belongs to the stages prior to the DGGE, namely PCR and nucleic acid extraction. Thus, all the troublesome features of sampling, DNA (or RNA) extraction, reverse transcription (if employing RNA extraction), PCR primer design, PCR conditions, and PCR cleanup bear some thought when troubleshooting DGGE problems. Other limitations with the DGGE method are; difficulties comparing bands from different gels due to gel to gel variations, different populations might have the same position in the gel due to similar GC content, and that it only works well with short fragments (<500 bp) thus limiting phylogenetic characterization.

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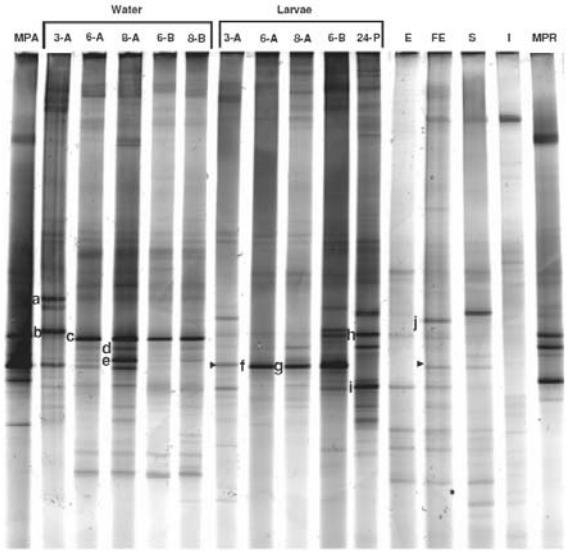


FIG. 1. Analysis of 16S rDNA fragments recovered by DGGE from water in scallop tanks, and bacteria associated with scallop larvae

A. DGGE analysis

Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting method that separates polymerase chain reaction (PCR)-generated DNA products. The polymerase chain reaction of environmental DNA can generate templates of differing DNA sequences that represent many of the dominant

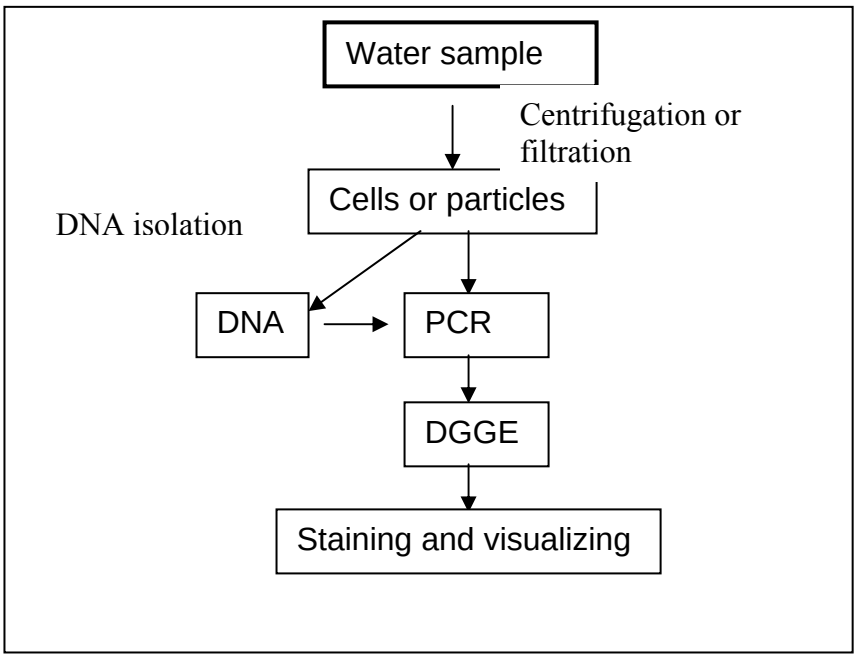
microbial and viral organisms. However, since PCR products from a given reaction are of similar size (bp), conventional separation by agarose gel electrophoresis results only in a single DNA band that is largely non-descriptive. DGGE can overcome this limitation by separating PCR products based on sequence differences that results in differential denaturing characteristics of the DNA. The technique exploits (among other factors) the difference in stability of G-C pairing (3 hydrogen bonds per pairing) as opposed to A-T pairing (2 hydrogen bonds). A mixture of DNA fragments of different sequence are electrophoresed in an acrylamide gel containing a gradient of increasing DNA denaturants. In general, DNA fragments richer in GC will be more stable and remain double-stranded until reaching higher denaturant concentrations. Double-stranded DNA fragments migrate better in the acrylamide gel, while denatured DNA molecules become effectively larger and slow down or stop in the gel. In this manner, DNA fragments of differing sequence can be separated in the acrylamide gel. Each band theoretically representing a different microbial population present in the community. Once generated, fingerprints can be uploaded into databases in which fingerprint similarity can be assessed to determine microbial/viral structural differences between samples.

B. Preparing samples for the DGGE analysis

Efficient, nonselective methods to obtain DNA/RNA from the environment are needed for rapid and thorough analysis of viral and bacterial community diversity. Principally there are two ways of preparing samples that can be used for PCR amplification. The first step is to isolate the cells/particles from the sample. Thereafter the cells/particles might be used directly in the PCR mastermix and lysed during the denaturation steps. The other method is based on extracting DNA/RNA from the cells/particles prior to PCR. The nucleic acids can then be used as template in the PCR reaction. Successful characterization of microbial/viral communities in the environment might sometimes require efficient extraction of the nucleic acids from environmental

samples and an adequate purification from the co-extracted contaminants that otherwise might inhibit the PCR reaction.

PCR-DGGE



2.0 Procedure

A. Polymerase Chain Reaction (PCR)

A few examples can be seen in Table1:

Table 1. Primers used for amplification of PCR products

Template	**References**
Bacterial 16S rDNA	G. Muyzer, E.C. de Waal and A.G. Uitterlinden. 1993.
Eukaryote 18S rDNA	van Hannen, E. J., M. P. van Agterveld, H. J. Gons, and H. J. Laanbroek. 1998.
EhV- Major Capsid Protein	Schroeder DC. J. Oke, M.Hall, G. Malin, and W.H. Wilson. 2003.
Algal-DNApolymerase	Short S.M. and C.A Suttle, 2002 & 2003

Important: The majority of PCR products generated for DGGE utilises a GC-tail attached to the 5` end
GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3`

B. Denaturant gradient gel elechtrophoresis (DGGE)

DGGE gels will be poured and run to separate similarly sized PCR products. You will create gels by combing two solutions containing acrylamide (structural material) and differing amounts of denaturants (urea and formamide) to form a gradient of denaturant in which double stranded DNA fragments of differing sequence will be denatured during electrophoresis. The gel will be stained and visualized to reveal band patterns that can be used to determine the similarity of sampled microbial communities.

I. Building the gel assembly

- Using lint-free tissues, wash glass plates, spacers and combs thoroughly with 70 % ethanol. Do not use soap or harsh abrasive cleaning materials to clean any of the equipment. If the materials are cleaned diligently, there is no need to use any detergents. A simple water rinsing will suffice followed by 70 % ethanol.
- Assemble the gel sandwich by placing the small glass plate on top of the large plate, being sure to correctly place a 1 mm spacer along each edge of the plate assembly. Grease both sides of the spacers with as little as possible silicon grease to cover the full length of the spacer but only a quarter of the spacer width. Attach the plate clamps (tight enough to hold everything together) and place the entire assembly into the rear slot of the pouring stand. Inspect the plate assembly to ensure that the two glass plates and the spacers form a flush surface across bottom of the assembly. If not, breaches in the seal of the plate assembly with the bottom of the pouring stand will result in leaking gel solutions.

- Place the glass plates and spacers together with the sandwich clamps in the casting stand in which a rubber strip is placed at the bottom to prevent leakage.

- Check the gradient maker and flush with milliQ. Empty pump tubing and attach pipette tip at the outlet tube to the top-middle of the gel chamber.

II. Prepairing the gel

• Wear gloves throughout the entire protocol. Acrylamide and formamide are toxic. Use caution when working with these materials.

To set up a new DGGE (new primer set, new sample type/habitat) we usually start with a relatively broad gradient (20-80% denaturant). However, we then focus to the area of interest which should include the highest and lowest bands in different samples. For example, the Eukaryotic DGGE described by Van Hannen et al. (1998) as well as for the bacterial DGGE described by Muyzer et al. (1993) we use a denaturant gradient of 30 to 55%.

- Use the following table to determine the appropriate composition of the denaturing gradient gel. You will make two solutions of 14.5 ml volume each; a "low" denaturant concentration solution and a "high" denaturant concentration solution. For example, if you wish to make a 30–55% gradient, then you would make a 30% (low) solution and a 55% (high) solution based on the reagent volumes in Table 1.

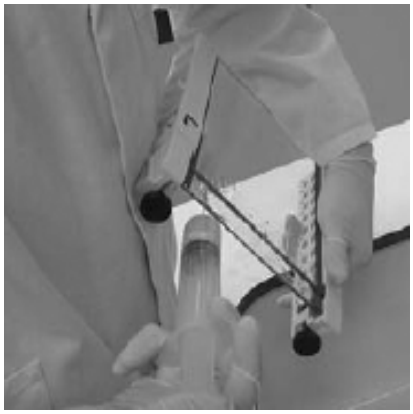
Table 3. DGGE gel composition.

% Denaturant	Solution A (0%)	Solution B (100%)
0	14,5	0
15	12,3	2,18
20	11,6	2,9
25	10,9	3,6
30	10,2	4,3
40	8,7	5,8
45	7,8	6,5
50	7,25	7,25
55	6,6	7,9
60	5,08	9,43

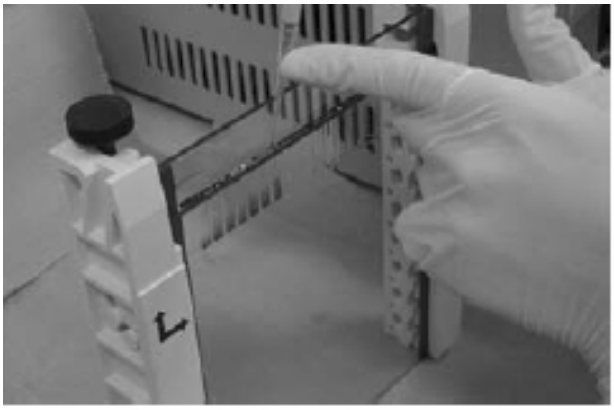
- Add 145 ul 10 %APS and 7,25 ul TEMED into each solution and swirl gently to mix. These reagents begin the polymerization of the acrylamide. At this point you will have approximately 15 minutes to pour the gel.
- Make sure the pump is off and the gradient maker-channel is closed (handle up). Pour the acryl-high in the right leg of the gradient maker (at the pump-side) and the acryl-low in the left leg. Stirring bean on. Simultaneously: Start the pump (5 ml/min) and move the handle of the gradient maker to horizontal position (channel open). The gel-chamber fills slowly. Use approx 4-5 min to fill the gel.
- Empty the tubing and flush thoroughly with milliQ.
- Place the comb to the right position.
- The gel needs approximately 2h to polymerize. The gel can be kept at 4^{o}C until the next day.

III. Electrophoresis

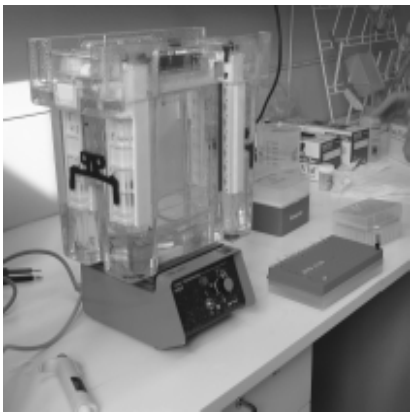
- Prepare approximately 7 L of 1X TAE and fill the buffer chamber. Put about 0.5 L aside for later use. Place the tank on a magnet stirrer and add a magnet in the bottom of the tank. Preheat the buffer in the DCODE apparatus to 60°C; this will take about 2 hours.
- Flush each well with buffer to remove any unpolymerized acrylamide. Failure to do this might result in uneven well floors and unresolved bands.



- Flush each well with buffer again and load approximately 15-20 µl of the PCR product containing loading dye to each well. The volume loaded depends on the success of the PCR and the number of expected products. The samples are most easily pipetted into the slots when the gel is standing at the bench. Load a marker on each side of the gel along with the samples. (For determination of band positions and comparability of gels).



- Attach the gel-plates in the core assembly. Loosen the clamps a quarter counter clockwise to prevent breaking of the sandwich clamps (due to heat-expansion). Then place the core assembly into the heated buffer in the tank.



- Start and check the circulation of buffer, start the stirring, and run at 60 V (about 20 mA for one gel) for 19h.

IV. Staining

- When the electrophoresis is complete, take apart the apparatus and remove the glass plates from the gel clamps. Carefully separate the plates, leaving the gel exposed on the large plate. Use the edge of the small plate to trim the well walls, but be sure to leave the left-most wall slightly higher than the others for use as a reference. Also, trim off any portions of the gel that do not contain used lanes. For easy manipulation, the gel should be transferred to, stained on, and transported on plastic wrap.
- Stain the gel for 30 minutes in 50 ml of SYBERGold DNA gel stain (Molecular probes) diluted 1:10,000 in SdH_2O. Remember, GelStar binds to nucleic acids therefore it is important to minimize

contact with skin, so wear gloves (powder-free). The container for staining should be plastic and not glass.

- Slide the gel off of the plastic wrap onto a UV transilluminator and view the gel.
- Bands of interest might be excised from the gel by using a tip (1 ml) and placed in an eppendorf tube with 20 µl SdH_2O over night at 4^oC. The extracted DNA can be used in a second PCR and sequenced.

Material and solutions for DGGE

- **Plate sandwich materials**
  16 x 16 cm glass plate
  16 x 14 cm glass plate
  2 - 1 mm spacers
  2 - plate clamps
  Pouring stand
  Foam gasket
  Well comb

- **Solution A** (250ml, 0% denaturant):
  50 ml 40% Acrylamide stock solution (bis-acrylamide gel stock solution 37:5:1; Bio Rad Laboratories)
  2.5 ml 50 x TAE
  Adjust to 250 ml with sdH_2O

- **Solution B** (250 ml, 100 % denaturant)
  50 ml 40% Acrylamide stock solution
  2.5 ml 50 x TAE
  105 g Urea
  100 ml Deionized Formamide (Pharmacia biotech)
  Adjust to 250 ml with sdH_2O

- **TAE buffer (50X)**
  20 mM Tris-acetate (pH 7.4)
  10 mM Sodium acetate
  0.5 mM Sodium- EDTA

- **10% Ammonium Persulfate Solution** (APS, 0,1 g in 1 ml of nanopure water. Make fresh when required or aliquot 0.5 ml into 1.5 ml microcentrifuge tubes and freeze until needed)

- TEMED (N,N,N',N' -tetramethylenediamine)
- SYBR Gold gel stain (Molecular Probes)
- DGGE apparatus;
  - Hoefer Scientific SE600
  - Bio-Rad
    - www.bio-rad.com
  - Ingeny
    - http://www.ingeny.com/
      http://www.ingeny.com/ingeny_brochure_eng.pdf
  - CBS Scientific
    - http://www.cbssci.com/
- Gradient maker
  - SG Gradient Makers (GE Healthcare)

- Peristaltic pump
- Power supply
- UV transilluminator and Gel Doc system
- Gel analysis products
    - BioNumerics (Applied Maths, Kortrijk, Belgium)
    - Quantity One (Bio-Rad)
    - DGGESTAT
    - Gelcompare II

3.0 References and useful information:

- Yahoo DGGE group http://groups.yahoo.com/group/dgge/.
  Net group were you can get practical help and information from other people working with DGGE for microbial ecology analysis.
- DGGE protocol: http://www.nioo.knaw.nl/CL/MWE/protocol_DGGE.pdf.
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Case study abstracts

Phaeocystis viruses

Corina P. D. Brussaard
Dept. Biological Oceanography, Royal Netherlands Institute for Sea Research (NIOZ)
1790 AB Den Burg, Texel, The Netherlands

Viruses can be important regulating factors for phytoplankton population dynamics. Bloom forming algal species with their high cell densities may allow a rapid propagation of lytic viruses, resulting in a strong direct control of the algal population by viral lysis. The genus *Phaeocystis* includes several bloom-forming species that are key algae in many ecosystems. The ecological significance of the genus is also related to its polymorphic life cycle with free living single cells and mucoid colonial stages. The single cells of *Phaeocystis* species are found sensitive to viral infection. The viruses infecting *Phaeocystis globosa* and *P. pouchetii* are diverse and so far all isolated viruses infecting these species belong to the virus family Phycodnaviridae.Viral infection of *Phaeocystis* impacts not only the host population dynamics, but also on the structure and function of the whole pelagic food web and on biogeochemical fluxes. Viral mediated mortality of *Phaeocystis* in natural ecosystems is a quantitatively significant process and comparable to grazing mortality. The various methodological aspects used for the above mentioned research will be discussed.

Chlorella viruses

Jim van Etten
Dept. of Plant Pathology, University of Nebraska

Paramecium bursaria chlorella virus (PBCV-1) is the prototype of a rapidly expanding group (family *Phycodnaviridae*, genus *Chlorovirus*) of large, icosahedral, plaque-forming, double-stranded DNA (310 to 370 kb) viruses that replicate in certain unicellular, eukaryotic chlorella-like green algae. In nature, the chlorella host is a hereditary endosymbiont of the cilliated protozoan *Paramecium bursaria* and while inside its protozoan host, the alga is protected from the viruses. The alga host can be grown in the laboratory in liquid and on solid media and since the viruses are lytic to the host, this led to the development of a plaque assay, which was the first virus infecting a photosynthetic host that could be assayed by a plaque assay.

ISOLATION AND CHARACTERISATION OF VIRUSES INFECTING MARINE EUKARYOTIC MICROALGAE

Keizo Nagasaki

*National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Japan**

So far, more than 17 viruses infecting marine eukaryotic phytoplankton have been isolated and their physiological, ecological, and genetic characteristics are illustrated at different levels of resolution. As far as looking at their unusualness and high diversity, it appears still valuable to isolate new host-virus systems and precisely examine the characteristics.

The flow of virus isolation procedure is very simple: screening, cloning, and maintenance. In screening viruses infectious to a certain algal species, it is strongly recommended to use multiple clonal isolates as hosts, because virus sensitivity spectra can differ at the strain levels. In many cases, exponentially growing cultures tend to be more sensitive to viral attacks than stationary phase cultures are. Microalgal viruses are included not only in seawaters but also in marine sediments; thus, sediments are also a promising resource for new virus isolation. When a viral infection is positively detected, the pathogen suspension should be cloned by >2 cycles of extinction dilution procedure, made free from bacterial contamination by filtration, and appropriately stored. Conditions required for stable preservation differ among viruses; hence, it is necessary to maintain the virus by repetitious transfer to fresh host cultures before establishing a suitable storage method. However, it should be noted that it might cause a loss of infectivity, which is presumably due to the increase of defective interfering particles.

In the present paper, characteristics of several viruses that are infectious to marine dinoflagellates, diatoms and raphidophytes will be also summarized (For details, refer to the below articles).

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Nagasaki, K. *et al.* Comparison of genome sequences of single-stranded RNA viruses infecting the bivalve-killing dinoflagellate *Heterocapsa circularisquama*. *Appl. Environ. Microbiol.* **71(12):** 8888-8894 (2005).

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Prophages: Dangerous Molecular Time Bombs or the Key to Bacterial Survival in the Oceans?

JOHN H. PAUL
Distinguished University Professor, College of Marine Science,
University of South Florida, 140 7th Ave S., St. Petersburg, FL 33702

Lysogeny is the process whereby a temperate bacteriophage establishes a stable symbiosis with its host. Known for some time from the study of bacteria important in the fields of medicine and the dairy industry, the ecological underpinnings of lysogeny are poorly understood as is its significance in the marine environment. This talk reveals the mysteries of lysogeny in the seas, starting with a primer of definitions, observations in cultivated temperate phage host systems, our current understanding of the significance of lysogeny in natural marine populations, the current state of marine prophage genomics, and a glimpse at a temperate metavirome from a tropical estuary. Finally, based upon independent observations, a unifying principle in the rationale for lysogeny is derived, as a means for bacterial survival in the oceans.

Viral community analysis.

Ruth-Anne Sandaa
Dept of Biology, Research group; Marine microbiology, University of Bergen.

I will show examples, both from field and mesocosm studies, on how we have applied Pulsed Field Gel Electrophoresis (PFGE) in combination with other methods (FCM, PCR, cloning and sequencing) in our studies of viral communities. We have investigated the viral dynamic related to the host community, and how it changes under different environmental factors, using PFGE for analysis the viral community and DGGE and FCM to investigate the dynamics in the host community. By using this approach we have discovered that the viral community correlates with changes in the bacterial and phytoplankton community structure, and that viral lysis is responsible for the termination of algal blooms. Recent estimates have shown that more than 99 % of marine bacteria (potential phage hosts) are unculturable using standard methods. Thus, when investigating genetically and functional viral diversity in environmental samples it is important to use cultivation- independent approaches. With PFGE it is possible to gain information about the dominant and presumably the most active viral populations in the samples, with no need for a culturable host. Compared to other cultivation-independent approaches (e.g. metagenome sequencing), PFGE also provides us information about the genome size of the viruses. Such information can be used to suggest the likely family that these viruses belong to. These viral populations might further be investigated by PCR using primers or probes against certain taxonomic groups of viruses or functional genes. Using this approach we have discovered a much higher diversity within certain viral taxonomic groups than previously reported. Likewise, we have detected functional genes (photosynthetic genes and phosphate sensing and acquisition genes genes) in a broad range of viral groups.

Viruses Infecting *Heterosigma akashiwo*

Curtis Suttle
EOS, Oceanography, Univ of British Columbia, 6270 University Blvd., Vancouver, BC, V6T 1Z4, CANADA

Heterosigma akashiwo is an icthyotoxic bloom-forming alga that is of particular concern in temperate waters of the Pacific. This case study will demonstrate the protocols that were used to titre, isolate, purify and characterize viruses infecting this alga. This will include some discussion of tangential-flow filtration, titering by most-probable-number (MPN) assay, cloning by end-point dilution and purification by centrifugation. In addition, data will be presented showing the distribution of these viruses in nature and their characterization.

DETERMINATION OF VIRUS-INDUCED MORTALITY IN MARINE SYSTEMS

Markus G. Weinbauer
Microbial Ecology and Biogeochemistry Group, Laboratoire d'Océanographie de Villefranche, Villefranche-sur-mer, france

Several methods have been developed to assess viral production and virus-induced mortality of heterotrophic prokaryotes, however, none of these methods has a level of acceptance as methods used to estimate primary and bacterial production. Nevertheless, results using different methods show often the same trends, although absolute values can differ. Assessing virus-induced mortality for primary producers is even more difficult. Most of the methods used to estimate virus-induced can also be applied in combination with an inducing agent to determine lysogeny in prokaryotes. Clearly, more intercalibration studies of different methods and development of new or refined methods are needed to obtain harder data on virus-induced mortality. Molecular tools are one avenue on this way.

Markus G. Weinbauer
Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-mer, France
wein@obs-vlfr.fr

Sampling for viruses across oceanic realms: where are you going and what are you doing?

Steven Wilhelm
Department of Microbiology, The University of Tennessee, Knoxville, TN, 37996-0845

As with any ecological scientific endeavour, perhaps the most critical aspect of the research is the proper collection, processing and transportation of the samples from the field site to the laboratory for final analysis. As part of the development of a plan of research, it is important to keep in mind the specifics of the hypotheses being tested and questions being addressed. As part of this case study, historical examples of successes (and significant failures) will be used to highlight the importance of the sample collection and handling process. Virus abundance and diversity across large spatial and temporal transects and comparisons between "paired" studies will be used to highlight how improper sampling can lead to bad data as well as improper interpretation.

METAGENOMICS AND THE MARINE VIRUS COMMUNITY

K. Eric Wommack
Delaware Biotechnology Institute,
Dept. of Plant and Soil Sciences, and Graduate College of Marine Studies,
University of Delaware, USA

Application of high-throughput DNA sequencing technologies to the analysis of whole microbial communities, i.e., microbial metagenomics, has truly begun to reveal the detailed inner workings of the proverbial microbial black box and ushered in the era of gene ecology. By and large, microbial metagenome sequencing efforts have focused on bacterioplankton; however, as compared to other classes of microorganisms, metagenomics may be best suited to examination of viral communities. Arguably, laboratory cultivation-bias is greatest for viruses; thus, known viruses likely represent less than $1/100^{th}$ of total viral diversity. Although a number of marker genes have been important to constraining the diversity of viral groups, there is no universal phylogenetic marker for viruses. Because of the small, coding dense genomes of viruses, viral community metagenome sampling should reveal genes which are active within the environment. The handful of characterized viral metagenomes examined to date reveal that viral communities contain an extraordinarily diverse number of genotypes and are the largest reservoir of unknown genes on Earth. As viral metagenomics is a nascent field and a number of new and cheaper technologies for high-throughput DNA sequencing will soon be widely available, methodological approaches to the metagenomic characterization of viral diversity are in flux. This paper will summarize the current state of viral metagenomics; outline the methodologies and bioinformatic analyses used in these studies; and highlight the future promise of metagenomic approaches to viral ecology.

Guest speaker abstracts

Insect viruses: Ecology, biotechnology and manipulation in the garden.

John Burden.
Centre for Ecology and Hydrology, Mansfield Road, Oxford.

Insects are susceptible to infection by a wide range of different viruses. These include examples of both DNA and RNA viruses, some of which show similarities to their mammalian counterparts and some of which are families unique to insect species. All these viruses are involved in complex interactions with their hosts the outcome of which can vary from a lethal infection to a state of near symbiosis. Insect viruses also interact with their host at the population level, with outbreaks of virus disease playing an important role in controlling the density of many host species.

Baculoviruses are large double stranded DNA viruses that are only isolated from invertebrate species, most commonly from butterfly and moth larvae. They are characterised by the virus particle being embedded within a large proteinaceous matrix (occlusion body) which protects the virus from degradation in the environment, and they have been extensively exploited for both biotechnology and pest control applications. Genetically modified (GM) baculoviruses have been created in which insect selective toxins are expressed during infection resulting in significantly improved crop protection. However, concern about how they may persist in insect populations and how they may compete with wild-type viruses has fuelled intensive research in this area. This research is revealing just how complicated virus ecology can be, and the dangers of extrapolating from simple experiments.

One of the main areas of interest that has come out of this research in baculovirus ecology is the prevalence of persistent infections in many different host-virus systems. These infections are vertically transmitted and the host insect suffers no apparent cost. The virus retains the ability to revert to a lethal infection, although what triggers this in unclear. Persistent virus infections in insects are not unique to the baculoviruses and although generally symptomless, they can have devastating effects on insect populations when other factors are involved. This is highlighted by the complex interaction between honey bees, their viruses and the *Varroa* mite.

Evolutionary pressures on marine viruses

Nick Mann
Department of Biological Sciences,
University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, U.K.

The talk will examine the evolutionary pressures on marine viruses taking into account theoretical studies and evidence from other host-parasite systems.

Poster abstracts

UNLOCKING THE SECRETS OF GIANT VIRUSES

MJ Allen and WH Wilson
Plymouth Marine Laboratory, Prospect Place, Plymouth, Devon, PL1 3DH

Viruses are the most abundant particles in the oceans with up to 100 million found in a single teaspoon of seawater. We isolated a giant virus that infects a chalk-covered algae that forms beautiful oceanic blooms and soaks up billions of tonnes of carbon dioxide. Incredibly, during infection the virus is thought to control climate by producing a gas that helps clouds to form. The genome of this virus contains 473 genes; this is enormous if you consider a devastating virus such as HIV contains only 9 genes. It is the largest algal virus ever sequenced. Most of the genes have never been found in a virus before. For example, it has a group of genes that may slow down the ageing process of the infected cell by keeping it healthy for as long as possible. A compound called ceramide is produced, a key component of anti-ageing creams. Ceramide can control programmed cell death and may have applications in anti-cancer therapies. We are using gene chips to study how genes are controlled in this amazing virus. Among our many discoveries is a distinct group of genes, totally unknown to science, which are switched on in the early stages of infection. This cluster of novel genes is essential for virus to infect the unfortunate cells, yet their function is a mystery. We aim to solve this mystery and unlock the many other secrets contained within this amazing giant virus.

Virus and protists - competing for prokaryotes in an oligotrophic marine coastal system

Julia Boras

Viral lysis and protists grazing are the main factors controlling prokaryotic losses in aquatic systems. Recent studies showed that in oligotrophic systems, viral prokaryotic lysogenic infection and losses due to protists dominate over prokaryotic lytic infection. We tested during a seasonal cycle in an oligotrophic coastal area in the NW Mediterranean Sea whether: 1) virus lysogenic infection prevailed over lytic infection on prokaryotes and 2) protists are the main responsible of prokaryotic mortality. Results showed that prokaryotic and viral abundances did not covariate over the year, and no significant correlation was found between prokaryotes and protists. Prokaryotic lytic infection appeared to be more important than lysogeny during almost the whole year; lysogeny was detected only in four months. Prokaryotic losses due to grazers were larger than losses due to viral infection when viral production was low and lysogeny was detected. Hence, this study shows that virally caused mortality of prokaryotes can be as or more important than bacterivory, also in oligotrophic marine system.

EFFECT OF BLACK CARBON ON PHAGE - HOST INTERACTIONS

Raffaela Cattaneo

Black Carbon (BC) represents a refractory and chemically complex product of incomplete combustion of fossil fuel and biomass. BC is ubiquitous and is introduced into the ocean through several ways (wet and dry atmospheric deposition, river-runoff and resuspension). Although it constitutes a substantial fraction of the pelagic organic carbon pool, its impact on aquatic ecosystem is poorly studied. Preliminary experiments carried out with Standard Reference Material in sea water showed that the microbial community responded to BC addition. Bacterial production and abundance increased with higher BC concentration, while viral abundance was negatively correlated to BC and showed in particular a negative relation with particle attached bacteria. One of the aims of this doctoral project is to investigate the effect of BC on viral life cycles to understand the role of BC on the interactions between phages and bacteria. Research is needed to determine whether the observed viral decay is due to a direct effect on phages (e.g. inactivation) or whether BC particles protect bacteria and thus prevent new infections. Bacterial and viral abundance on BC will be quantified using a suite of techniques such as FCM, LSM and SEM as well as removal of cells and viruses from particles. It will also be tested whether BC affects viral turnover and percent lysogeny and how effects on viruses influence bacterial production and diversity.

Phages which infect the marine cyanobacterium *Acaryochloris marina*.

Yi Chan
University of Warwick

The photosynthetic cyanobacterium, *Acaryochloris marina* was discovered in 1996 and was found to contain mainly chlorophyll d, which allows it to harvest far red light and thus live in light-depleted habitats. Nothing is known about the relationship between *Acaryochloris* and phages as none have previously been isolated from this organism. It is known that the photobiology of other cyanobacteria (*Synechococcus* and *Prochlorococcus*) is influenced by phages as they carry key photosynthesis genes which are expressed during infection. They may also act as vectors for the evolution of their host bacteria. My research is to investigate the role of bacteriophages in the photobiology of Acaryochloris. I have therefore isolated phages which infect this bacterium. I shall characteris the morphology, host range, genome size and growth parameters of these phages. I will also obtain sequence information and examine whether host photosynthetic genes are present in their genomes.

PHAGE-MEDIATED GENE TRANSFER WITHIN FRESHWATER CYANOBACTERIA

L DENG & P. K. HAYES
School of Biological Sciences, University of Bristol, Bristol, U.K.

Cyanobacteria are important members of phytoplankton communities both in marine and freshwater environments. They are a significant source of primary production, forming the base of the food chain. Viruses are consistently the most abundant biological entities in aquatic systems, about 10^9 viruses per litre. Over the past two decades, it has been shown that viruses are important in controlling bacterial composition: some work has shown this to be true for cyanobacterial communities that are infected with cyanophage. There is increasing evidence that lateral gene transfer within cyanobacterial communities and populations has a role in generating novel phenotypes. Very little is known about phage-mediated gene transfer within freshwater species of cyanobacteria. There is also very little known about the role of cyanophage in regulating cyanobacterial population development and structure. The aims of this project are to isolate cyanophage able to infect strains of the freshwater cyanobacteria *Anabaena, Microcystis* and *Planktothrix*, to quantify diversity and dynamics within phage communities and to investigate the potential for phage mediated gene transfer within cyanobacterial populations.

MetaFunctions: Considering the Marine Phage

Melissa Duhaime
Max-Planck-Institute for Marine Microbiology, Celsiusstrasse 1, D-23859, Bremen, Germany

A novel approach to marine virus genomics is required to grasp the impact of viruses on the reservoir of genes directing the central metabolism of the world's oceans. The potential for marine viruses to act as "gene shuttles" between species is well known, though their impact on the gene pool of marine microorganisms has not been extensively studied. Metafunctions is an E.U. funded project, which aims to develop a system for the integration of genomic and ecological data. The goal of such an initiative is to detect and assign function to habitat specific gene patterns. The question at hand is whether the incorporation of marine phage to this project is necessary. If so, how should the incorporation be implemented? A comparative analysis of marine phage is the next step to extract and assign information to these highly diverse communities. Marine phage genome and metagenome data should be incorporated into the Metafunctions project.

Mean Green Viral Machine

Claudine E. Falla, Peter Bond, Roy Moate and Declan C. Schroeder

Virology has greatly expanded since Viruses first received their name in 1898; recent advances in technology and awareness have led to new innovative studies, which go some way to unravelling viral anonymity. Both NCLDVs and RNA viruses are highly abundant in the aquatic environment and have prompted increasingly bold theories on the origin of life, indicating a central role in evolutionary processes and demanding a place on the tree of life. This study characterises a new virus infecting the vegetative cells of the Ectocarpales *Streblonema*. Viruses infecting *Streblonema* dates back to the early 1970's, however, these viruses were never fully characterised. Initial analysis of our infected material reveals a unique replication strategy. This discovery in conjunction with the huge diversity seen in aquatic viruses has massive implications for the future of the planet, its productivity and life as we know it. It is not only the interpretation of our evolutionary past which is revealed by the increasing discovery of new viruses, but the global fate of the future.

VIRAL GENOME SIZE DISTRIBUTION IN PELAGIC AND BENTHIC ECOSYSTEMS

Manuela Filippini

The size distribution of virus-like DNA in natural samples was investigated in a number of marine, freshwater and terrestrial environments by means of pulsed field gel electrophoresis (PFGE). The community fingerprintings showed a number of cross-systems similarities with a general dominance of genome in the 30-45 kb, 50-70 kb and 145-200kb size fraction. However, systematic differences in community fingerprints were also observed between the investigated sites. Some virus genome sizes were present only in specific biotopes (e.g. lake water or agricultural soil), in specific ecosystems (e.g. a particular lake) or even in specific microhabitats (e.g. a particular sediment stratum). The data indicated that certain virus types are ubiquitous in aquatic environments and have moved between ecosystems, whereas other viruses seem to be specific for a given habitat.

VIRAL DIVERSITY ASSESSMENT AT THE GUARAPIRANGA RESERVOIR - SAO PAULO, BRAZIL

Manuela V. Gimenes[1]; Sônia M. Gianesella[2]; Maria Inês Borella[1], Adilson F. Nunes[3]; Patrícia Garrafa[1]; Telma A. Monezi[1] and Dolores U. Mehnert[1]
[1]Institute of Biomedical Sciences, University of Sao Paulo; [2]Oceanographic Institute, University of Sao Paulo; [3]Metropolitan Water Resources Department (SABESP).

In spite of the wideness of the Brazilian water sources there is a lack of information about the viral diversity in such waters. Some studies related to the eventual contamination of those waters by domestic effluent and consequently by human enteric pathogens, have been conducted through the last two decades. Guarapiranga reservoir is one of the largest reservoirs (630 km^2) for public supply of Sao Paulo city, assisting 3.4 million people. It is located inside the urban area, and, due to unplanned occupation, its surrounding has been undergoing a fast process of degradation and pollution. The purpose of the present study was to establish the first assessment on viral diversity at this environment. Five liters of surface water were collected at three distinct sites in August/2005. Samples were concentrated by filtration through a positively charged membrane (Zeta Plus 60S, AMF Cuno) followed by ultracentrifugation at 100,000 *x g.* The sediment was suspended in 0.1M phosphate buffered saline solution, pH 7.2, effecting an 8,000-fold concentration. Viral concentrates were detoxified by Vertrel-XF and subjected to electron microscopy (EM) and DNA extraction by using Trizol. Virus sequences were detected by PCR, using specific primers for *Phycodnaviridae* (algal viruses) and for *Myoviridae* (cyanophages) families and amplicons were subjected to DGGE analysis. Physical, chemical and other microbiological parameters were analyzed in the same samples. The concentration method adopted is a good alternative for such studies since both EM and molecular methods detected the two viruses families.

Diversity of marine *Prochlorococcus* and their co-occurring phages

Ellie Harrison, Plymouth Marine Laboratory

The diversity of communities of the marine cyanobacteria, *Prochlorococcus* and co-occurring cyanophages will be studied through amplification of molecular marker genes using PCR, combined with high throughput fingerprinting techniques. High resolution *Prochlorococcus*-specific PCR primers were developed for the *rpoC1* gene. The RNA polymerase core subunit gene, *rpoC1*, possesses greater genetic variation (70-99% sequence similarity) than the universal bacterial marker, 16S rRNA, (≥97% sequence similarity), thus providing the opportunity to study *Prochlorococcus* micro-diversity. PCR combined with RFLP analysis proved to be able to distinguish between *Prochlorococcus* strains with 98% *rpoC1* sequence similarity. RFLP enabled screening of large sample sets at high genetic resolution. Environmental DNA samples were collected from the Atlantic Ocean for screening. Clone libraries were constructed using the environmental DNA. RFLP analysis of clone libraries from the Atlantic showed that the respective *Prochlorococcus* populations were dominated by genetically different clones. The diversity of RFLP types seen in the Atlantic was also significantly different. Phylogenetic analysis revealed that the application of the newly developed *rpoC1* primers lead to the discovery of a previously unseen genetic diversity of *Prochlorococcus* with novel phylotypes.

Cyanophages have been isolated that infect *Prochlorococcus*. Phages have been demonstrated to affect the diversity of other cyanobacterial hosts. Therefore viral activity may account for the huge array of diversity observed in *Prochlorococcus* in the Atlantic Ocean. This project will provide a valuable insight into *Prochlorococcus* phage diversity and elucidate the influence they exert on host diversity.

COMPLEXITY OF INTERACTIONS BETWEEN LYTIC MARINE PHAGES AND THEIR *CELLULOPHAGA* HOSTS

Holmfeldt, K., Middelboe, M., Riemann, L.
Marine Microbiology, BoM, Kalmar University, S-391 82 Kalmar, Sweden.
karin.holmfeldt@hik.se, +46480447326

Phages influence the marine bacterial community by infection and lysis of bacterial populations. Lytic infection is commonly regarded as host specific and has the potential to regulate dynamics of individual bacterial populations; however, the specificity of this interaction is still largely unknown. To examine phage-host dynamics we isolated 21 strains of *Cellulophaga baltica* (*Bacteroidetes*) and 45 phages infecting these. The similarity among the strains was examined by colony morphology and by sequencing of the 16S rRNA gene and the internal transcribed spacer region. The phages were differentiated according to genome size by using pulsed field gel electrophoresis, RFLP-patterns, plaque morphology, and cross-test infectivity patterns. The bacterial strains showed high genetic similarity; e.g. more than 98% 16S rDNA similarity, but differed more on morphological characteristics. The phages showed large variations in plaque morphology and in genome size, ranging from 28 kb to more than 240 kb. The phages differed widely in host range, ranging from phages infecting all strains to phages infecting only two strains. Interestingly, despite the high genetic similarity of the bacterial strains, all showed unique infectivity patterns with respect to the 45 phages. These results illustrate the great complexity of marine phage-host dynamics and emphasize the necessity of including strain variation within bacterioplankton species in our perception of phage-host dynamics.

The evolution of Drosophila's antiviral immune system

Frank Jiggins

Viruses are important pathogens of wild Drosophila. Within natural populations there is considerable genetic variation both in the susceptibility of flies to viral infection. Some of this variation is caused by a mutation in a gene called Ref(2)P that is involved in the activation of a transcription factor in the fly's immune system. This mutation confers makes flies resistant to a natural pathogen called the sigma virus. The mutation is only about 1000 years old, which coincides to the date when the sigma virus itself spread through fly populations. Another important component of antiviral immunity is RNAi, which acts by degrading viral RNA. Antiviral RNAi genes are the fastest evolving component of the fly's immune system, and they are subject to strong directional selection that results in repeated selective sweeps. Together, these results support the hypothesis that there is an ongoing arms-race between RNA viruses and host resistance. To date there has been a dearth of studies that distinguish alternative models of host-parasite coevolution. Our data support a model of host parasite coevolution in which novel resistance mutations arise and spread to fixation due to natural selection. There is no evidence of frequency dependant selection maintaining ancient polymorphisms of resistant and susceptible alleles as predicted by many other models of host-parasite coevolution.

Molecular analysis of virus-induced bloom termination of the haptophyte *Emiliania huxleyi* strain 1516

Jessica Kegel[1], Mike Allen[2], Willie Wilson[2], Katja Metfies[1] und Klaus Valentin[1]

[1]*Alfred-Wegener-Institut Bremerhaven*
[2]*Plymouth Marine Laboratory (UK)*

Emiliania huxleyi is a cosmopolitan haptophyte that significantly contributes to global carbon cycling. Frequently occurring massive blooms of this algae in the oceans can be terminated by viruses. An expressed sequence tag (EST) approach was used to analyse the infection of *E. huxleyi* infected with the virus EhV86. cDNA libraries were constructed before, and 6, 12, and 24 h after infection, respectively, with the virus and 1000 – 2000 clones were sequenced from each library. From the nature and abundance of sequences in the library we could deduce the partial transcriptome of both, host and virus, through the infection process and draw conclusions on the infection mechanism. Very soon after infection the algal transcriptome changed significantly although only few viral transripts were present. After 24 h viral transcripts were most abundant. Viral transcripts found encode proteins involved in protein degradation, nucleic acid degradation, transcription and replication.

Fear of the Dark – Turn on the LAMP
Visualization of infected Algae using Loop Mediated Isothermal Amplification (LAMP-PCR)

Jens Borggaard Larsen, Aud Larsen, Ruth-Anne Sandaa, Runar Thyrhaug, Ana Paulino and Gunnar Bratbak
University of Bergen, Department of Biology, Jahnebakken 5, 5020 Bergen, Norway
Jens.larsen@bio.uib.no

Although several studies have indicated a possible role of viruses in termination of algae blooms, there is a paucity of data regarding viral impact on the growth dynamics of non bloom forming algae species. During the PeECE III mesocosm experiment that took place in May 2005, we tracked different populations of algae viruses using a combination of flow cytometry, pulse field gel electrophoresis, and degenerated PCR primers targeting the virus major capsid gene sequence, in order to correlate these data with the presence of algae species. As such, we identified three viruses tentatively assigned to the Phycodnaviridae family along with viruses infecting *Emiliana huxleyi*. One of these three viruses had a 100% homologue capsid sequence to a previously isolated virus infecting the haptophyte *Chrysochromulina ericina*, while the other two grouped together with viruses infecting *Heterosigma akashiwo*, and the chlorella virus, PBCV-1. We where following able to deduce a similarity between the patterns of some algae and infecting viruses. However, a closer description of the system stranded due to methodological hindrances in quantification of specific algae species, estimating number of infected cells, and counting specific free viruses. This let us to search for molecular methods allowing measurement of these factors. As a fast and inexpensive method, we tried to adapt the method of in situ PCR to the problem of quantifying infected cells. However, the high temperature necessary for denaturing DNA during PCR cycling, and the general small molecular size of the amplified fragments, tended to lead to cell loss and leaking of the product from the cells, the latter causing unspecific colouring. As such we are currently trying to develop a new approach to this problem, by applying the emerging technique of Loop Mediated Isothermal Amplification PCR (LAMP-PCR). This technique is promising as an alternative to in situ PCR, while it amplifies at general low constant temperatures, as it has proven to be highly sensitive, and since it generates high molecular weight DNA that would resist leaking of product from the cells.

Viruses: Agents of coral disease?

Willie H. Wilson[1,2], **Jayme Lohr[1]**, Joanne E. Davy[3], Isobel Francis[2,4], Cornelia Muncke[2,4], Sarah Burchett[2,4], Amy Dale[2,5,6] Piers Davies[2], Ove Hoegh-Guldberg[3] Simon K. Davy[7]

[1]Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth, PL1 2PB (current address)
[2]Marine Biological Association of the UK, Citadel Hill, Plymouth, Devon, PL1 2PB.
[3]Centre for Marine Studies, University of Queensland, Brisbane, QLD 4072, Australia.
[4]Institute of Marine Studies, University of Plymouth, Devon, PL4 8AA.
[5]Department of Biological Sciences, University of Plymouth, Devon, PL4 8AA.
[6]Department of Zoology, Ecology and Plant Science, University College Cork, Lee Maltings, Prospect Row, Cork, Ireland (current address)
[7]School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington, New Zealand.

The potential role of viruses in coral disease has only recently begun to receive attention. Here we describe our attempts to determine whether viruses are present in thermally-stressed corals (*Pavona danai*, *Acropora formosa* and *Stylophora pistillata*) and zoanthids (*Zoanthus* sp.), and their zooxanthellae. Heat-shocked corals produced numerous virus-like particles (VLPs), that were evident in the animal tissue, zooxanthellae and the surrounding seawater; VLPs were also seen around heat-shocked freshly isolated zooxanthellae. A wide range of different virus morphologies was observed suggesting that virus infection is commonplace in reef corals. The release of VLPs was again apparent when flow cytometry was used to enumerate release by heat-stressed *A. formosa* nubbins and zooxanthellae. Our data support the infection of reef corals by viruses, though we cannot yet determine the precise origin (i.e. coral, zooxanthellae and/or surface microbes) of the VLPs seen. Considerable work is still required to fully characterise these viruses and to determine their infection mechanism, but the potential for viruses to infect reef corals is clear and such infections may play an important role in determining the health of reef systems in the face of global climate change.

MOLECULAR ECOLOGY OF COCCOLITHOVIRUSES: GIANT MARINE VIRUSES THAT KILL THE BLOOM FORMING MICROALGA *Emiliania huxleyi*

Joaquin Martinez Martinez, Declan Schroeder, Michael J. Allen, Matthew Holden, Douglas Roy, Gunnar Bratbak & William H Wilson

Viruses play an important role in the life of plankton populations. Phytoplankton are at the base of the food web and the microbial loop in the oceans. Consequently, the viruses that infect them are likely to significantly affect the structure, function and biogeochemical cycling of marine food webs. *Emiliania huxleyi,* a marine microalgae that forms dense blooms in coastal and mid-ocean surface waters, is an important species with respect to past and present marine primary productivity, sediment formation and climate. We have sequenced the genome of an *E. huxleyi*-specific virus (EhV-86), generated a microarray for EhV-86, assessed its diversity in natural communities, determined its evolutionary position and investigated the role of a virus-encoded phosphate permease. All this data will be summarised in our poster.

Distribution and abundance of shiga toxin indicators in beach waters

Jayme Dylewski, Erin Lenz, Cody Smith, Denise Thompson, and **Steven Mauro**
Department of Biology, Mercyhurst College, 501 E. 38th Street, Erie, PA 16546, Phone: (814) 824-3619, e-mail:smauro@mercyhurst.edu

The shiga toxin gene produces a protein that is pathogenic to humans, and can lead to severe gastrointestinal illness. One of transmissible agents of shiga toxin is enterohaemorrhagic E.*coli* (including strain O157:H7), which harbors a bacteriophage that expresses shiga toxin upon lysogenic induction. Waterborne outbreaks of shiga toxin dependent sickness is a recurring problem in both developed and under developed countries. This work focuses on examining the interrelationship of the three critical elements required for shiga toxin dissemination in an aquatic ecosystem; the shiga toxin gene, enterohaemorrhagic E.*coli*, and bacteriophages that carry the shiga toxin gene. Using real time PCR coupled with standard microbial techniques, we have positively identified and quantified shiga toxin and enterohaemorrhagic E.*coli* DNA in a number of beach water samples. The relative abundance and distribution of both are variable, and not always correlated with one another. Current efforts are focusing on isolation and quantification of bacteriophages which carry the shiga toxin gene.

This poster is not presented as a poster, but is available as A4 handouts

Molecular Tools for *Microcystis* spp. Monitoring in Drinking Water Production and Raw Water Sources

Sandra M. Mesquita[1,2], Rute Miguel[1], Margarida P.Reis[1], Rachel T. Noble[2]
[1]Laboratório de Microbiologia, FCMA, Universidade do Algarve, Portugal, EU
[2]Institute of Marine Sciences, University of North Carolina at Chapel Hill, NC, USA

Occurrence of potentially toxic *Microcystis* spp. blooms represents a major concern for local and governmental agencies involved in water quality management, whether for recreational use or drinking water production. Direct microscope counts of picocyanobacteria may be misleading, often calling for taxonomic confirmation. To overcome such problems, molecular tools such as semi-nested PCR and fluorescence *in situ* hybridization (FISH) have been applied to environmental and treated drinking water samples collected both in southern Portugal (EU) and eastern North Carolina (USA). The aim of this work was to test the developed protocol on very diverse samples, including samples collected following each of 5 different processing steps of drinking water treatment. Collected samples were concentrated on mixed-cellulose membranes or polycarbonate membranes (0.45 um pore size) and were extracted using either the Dynabeads® DNA DIRECT™ Universal or the UltraClean™ Soil Clean Kit. Semi-nested amplification protocols were used to check for presence of *Microcystis* spp.. Optimization of PCR conditions and limits of detection were determined using raw water from different sampling sites spiked with serial dilutions of a *Microcystis aeruginosa* culture. Detection of *Microcystis* spp. was achieved in environmental samples containing a minimum of 1000 cell/ml, as checked by total cyanobacterial counts using proflavine dye and specific FISH counts using Alexa 350. Spiking of water samples lowered the detection limit to 100 cells/ml in samples collected from five different phases of the treatment process for drinking water production. FISH counts were easily applied to environmental samples, but lack reliability when used for treated water, because of interference of water treatment byproducts and the fluorescent signal. PCR amplification proved to be useful to monitor the efficiency of different treatment steps in removing *Microcystis* spp. from water. Given this, we are developing an optimized quantitative PCR assay that has the potential to be used as a powerful tool in monitoring cyanobacteria in raw and treated water sources.

Viral ecology in the floodwater of a Japanese rice field

Natsuko Nakayama, Mami Okumura, Katsuhiro Inoue, Susumu Asakawa, and Makoto Kimura

Virus is the most abundant biological component in marine and freshwater environments. And many studies have indicated the ecological importance of viruses in the primary production and microbial food web in marine environments. Repeated cycling of organic materials in the bacterial-viral loop causes the bacteria to be efficient sinks for C and regenerations of N and P in the environment where viruses are important agents of microbial mortality.
However, precise enumeration of viruses in soil environment has been unsuccessful due to firm viral adsorption to soil particles. Floodwater in rice fields is an aquatic environment and the environment free from viral adsorption to soil particles. However, no study has been conducted on the viral ecology in the floodwater. As the floodwater ecosystem in rice fields is directly influenced by field management, this presentation enumerated the viral abundance in the floodwater of a Japanese rice field under a long-term fertilizer trial to evaluate the effects of the type of fertilization and the growth stage of rice plants. This is the first study on the viral ecology in the floodwater.
The study field was located in the Aichi-ken Anjo Research and Extension Center, Central Japan (latitude 34.8° N, longitude 137.5° E). The field has been subjected to a long-term fertilizer trial since 1925. Four plots [a plot without fertilizers, a plot with chemical fertilizers, a plot with chemical fertilizers and lime, and a plot amended with chemical fertilizers and 22.5 tons ha^{-1} compost (CM plot)] were chosen in this study (3.6×7.3 m^2 to 3.6×9.1 m^2). Rice plants (*Oryza sativa* L. cv. Nihonbare) were cultivated in the respective plots under conventional management. Midseason drainage and the drainage for harvesting rice were performed in mid July and on September 28, respectively.
Floodwater was collected at the midway of four sides of each plot along the footpaths once every week or two weeks during the cultivation period (11 times from June 15 to September 22). The sample water was passed through a 47 um sieve, fixed with glutaraldehyde, and kept at -80 ºC until viral and bacterial enumerations. Viruses were first extracted with 3.6 % nutrient broth (NB) solution under ultrasonication for 2 to 3 min for desorbing viruses from suspended particles. Then, they were separated from suspended particles by centrifugation (8000 rpm for 10 min) and filtration with 0.45 um Nuclepore filter successively. Viruses were stained with SYBR Green I for 15 min at 80 ºC, and enumerated with a fluorescent microscope. Separately, viruses in free form were determined after filtration with 0.45 μm Nuclepore filter without extraction with NB solution. Bacterial abundance in each floodwater sample was enumerated with SYBR Green I for 30 min at room temperature. In addition, composite fresh water samples from four sites were diluted sequentially and subjected to MPN method for enumerating bacteriophages against 18 bacterial strains that had been isolated from the floodwater of the rice field (6 α-, 2 β-, and 2 γ-Proteobacteria, 4 CFB members, a *Bacillus* sp. and 3 high GC Gram-positive bacteria).

Bacterial abundance in the floodwater ranged in the order from 10^5 to 10^8 mL^{-1}, and there was no significant difference in bacterial abundance among the plots. It tended to increase with time after transplanting first until the beginning of July and was more numerous on June 28 and July 7 than on the other sampling time. Viral abundance in the floodwater was significantly correlated with the bacterial abundance, and ranged in the order from 10^6 to 10^9 mL^{-1}. Virus-to-bacterium ratios fell within the range from 0.11 to 72, and they tended to be larger during the middle growth stage of rice plant after midseason drainage when the viral abundance was smaller in the floodwater. Both viral and bacterial abundance also took a significant relations with the absorbance of water sample at 660 nm, indicating their adsorption to suspended particles as main way of presence in the floodwater. And viruses in free form (filterable with 0.45 μm Nuclepore filter) amounted to 10^6 to 10^7 mL^{-1} without any seasonal variation.
Fourteen to 73 % of floodwater samples contained more than a bacteriophage ml^{-1} against the bacterial isolates (35 % on average). Among the tested host bacteria, bacteriophages infecting three strains of *Sphingomonas* sp., *Enterobacter* sp. and *Cytophaga* sp. often amount to over 10^2 to 10^3 mL^{-1} in the floodwater of the rice field. In general, bacteriophages tended to be abundant after midseason drainage.

Dynamics in virioplankton production in the North Sea

Verónica Parada and G.J. Herndl
Dept. of Biological Oceanography, Netherlands Institute for Sea Research (NIOZ), P.O. Box 59, 1790 AB Den Burg, The Netherlands
Key words: viral diversity, PFGE, North Sea

Viral diversity has been studied in a variety of marine habitats and spatial and seasonal changes have been documented. As most of the bacteriophages are considered host-specific, they likely affect the numerically dominant prokaryotes more than less abundant phylotypes. We hypothesized that viral infection and consequently lysis occurs in pulses with only a few prokaryotic phylotypes lysed at any given time. Thus, we propose that the newly produced viruses resemble only a fraction of the viral diversity present in situ at any given time. We tested this hypothesis by determining the viral diversity newly produced by bacterioplankton and compared this with the in situ virioplankton diversity.
Virioplankton diversity was assessed by pulsed field gel electrophoresis (PFGE) in three distinct areas of North Sea surface waters during the spring and summer 2003 and 2005. Virioplankton diversity was fairly stable. Viral diversity produced by the indigenous bacterioplankton, however, exhibited day-to-day variability with only few bands produced at any given time. These bands frequently matched bands of the in situ virioplankton but occasionally, bands appeared which were not present in the band pattern of the in situ virioplankton community. These new bands would indicate infection of bacterioplankton previously not infected by specific viruses. Our results indicate that viral infection and lysis are rather dynamic processes and might hence effectively regulate and maintain bacterioplankton diversity.

The ghosts of viruses past: A quest to understand bloom evolution.

Steve Ripley, Declan Schroeder

The coccolithophore, *Emiliania huxleyi* is thought to be the main species responsible for global calcium carbonate production. As such this organism plays a fundamental role in global CO_2 cycling and the carbonate chemistry of the oceans. To evaluate the response of this functional group to the effects of climate change or to changes in virus communities known to play a pivotal role in bloom termination, we undertook a feasibility study to determine whether a retrospective approach could be used on archived coccolithophore datasets.

Influence of carbon availability on soil viral abundance

Sharath Srinivasiah & K. Eric Wommack
Department of Plant and Soil Sciences, University of Delaware

In contrast to better studied aquatic environments, little is known of the abundance, diversity and ecological role of viruses within soil microbial communities. It is well known that changes in the composition and abundance of available organic carbon can dramatically influence the diversity and activity of soil microbial communities. Thus, as an initial step towards establishing viruses as dynamic members of soil microbial communities, microcosm studies were designed to investigate the effect of carbon enrichment and mineralization on the abundance of indigenous soil viruses. Three carbon sources (Chitin, yeast-extract, and methanol) were added to Delaware agricultural soil in individual treatments at a rate of 5 mg C g^{-1}. Un-amended soils at the same moisture content served as a control. Each microcosm was maintained at controlled conditions with moisture content of 20.7%, an average room temperature of 23±2º C, and aeration at each sampling interval. All the microcosms were treated with cycloheximide to avoid fungal growth.

As expected, respiration in the yeast extract treatment remained high throughout the one month experiment; and after 14 days, respiration in the chitin treatment reached similar high levels. Respiration in the methanol treatment was low and similar to that of the control. After one week viral abundance in the yeast extract treatment increased 7-fold to a peak abundance of ~3.5 X 10^9 viruses per gram dry weight (gdw^{-1}) soil. In the chitin treatment peak viral abundance was 5-fold greater than initial values and did not occur day until 14 which corresponded with peak levels of respiration. Viral abundance in the methanol treatment was consistently lower than the control demonstrating the selective influence of this carbon source. Interestingly, all treatments showed a brief spike in viral abundance at two days which may have been the result of increases in moisture content across all microcosms. These initial experiments are the first definitive demonstration that short term changes in microbial activity, i.e., shifts in C content and availability, can influence soil viral abundance. Thus, as with aquatic environments, viruses are dynamic members of soil microbial communities.

A novel method for analyzing infection mechanism of algal viruses: viral RNA transfection using gene gun

Tomaru Y, Mizumoto H, Nagasaki K

Recent studies revealed the existence of microalgal RNA viruses in marine environment and several host–virus systems were successfully made into culture. The infection mechanisms of these viruses, however, are scarcely understood. The particle delivery system, so called gene gun, is a device for introdicing any nucleic acids into cells. Briefly, gold particles coated with DNA or RNA are shot into cells, then the injected nucleic acids are partially released into the recipient cells to establish their transfection. This method is an easy transfection technique and applicable for analyzing animal and plant virus infection mechanisms. In this study, we aimed to apply and optimize the particle bombardment method to marine dinoflagellate *Heterocapsa circularisquama* and its infectious RNA virus, HcRNAV. This algal RNA virus comprises two distinct ecotypes having complementary intraspecies host ranges; then, we also analyzed the mechanism of the ecotype-specific infection of HcRNAV using this method. The results showed that the method is applicable to the transfection of *H. circularisquama* cells, and suggested that the intraspecies specificity of HcRNAV is determined by the upstream events of virus infection. To precisely inspect algal virus infection mechanisms at a molecular level, transfection of microalgal cells by means of particle bombardment method is considered to be a useful tool. This method is most likely applicable to study other microalgal host–virus systems with a suitable optimization.

Infectious hematopoietic necrosis (IHN) virus in wild and farmed salmonids in British Columbia, Canada

Garth Traxler
Department of Fisheries & Oceans, Pacific Biological Station, Nanaimo, British Columbia, Canada V9T 6N7

Infectious hematopoietic necrosis (IHN) virus is endemic in certain Pacific salmon species along the west coast of North America. In British Columbia, the virus is most frequently associated with sockeye salmon. The virus usually found in this species at the extremes of the salmon's life cycle. IHNV is carried asymptomatically in spawning adult salmon and can also be found in young fish prior to entering sea water where it can cause high losses in both cultured and wild stocks. Atlantic salmon were introduced into BC in the mid-1980's for aquaculture purposes. IHN has caused major economic losses to the aquaculture industry which are estimated at $ 200 million in lost sales. In the early 1990's high losses due to IHN virus occurred at several farmed salmon sites all involving Atlantic salmon. There have been two additional epizootics caused by IHNV which was determined by sequencing studies to have been introduced from wild fish or an unidentified marine reservoir. In all cases farm-to-farm spread occurred soon after the index case either by water movement, or transfer by equipment/personnel. The virus can remain viable for months in seawater under optimum conditions but is also readily inactivated by chemical and physical methods. In order to develop effective health management plans it is important to identify possible reservoirs and viral sinks and to be able to distinguish viable or infectious virus from genomic evidence.

Isolation and quantification of viral RNA in the Nissequogue River (New York)

Alexandra Valdés-Wochinger

As part of an effort to develop microarrays for the detection of RNA viruses, we attempted to isolate viral RNA from surface water samples collected from the Nissequogue River on Long Island, NY. The 1 - 20 L samples were prefiltered through a 40 μm plankton mesh and concentrated to a volume of 30 - 45 ml via tangential flow filtration using 0.2 μm and 100,000 NMWC membrane cartridges. Further concentration to about 2 ml was carried out with a Centriplus centrifugal filter device (Amicon). Direct counts performed by epifluorescence microscopy after SYBR-Gold staining indicated the presence of 10^4 - 10^9 vlps/ml in the concentrate. RNA was extracted and quantified by the QIAamp Viral RNA mini kit (Qiagen) and Quant-iT RiboGreen RNA Assay kit (Invitrogen). Virtually all extracted RNA appears to have been carrier RNA from the Qiagen kit. Additional RNA extraction protocols are being explored, but results suggest the possibility that RNA viruses may constitute a minority of those viruses present in Nissequogue River water and that much larger sample sizes may be needed.

The establishment of natural Daphnia-virus system for experimental evolutionary epidemiology

Pedro Vale
Institute of Evolutionary Biology, University of Edinburgh,

Research in our lab deals with the evolutionary causes and consequences of parasitism in natural populations. Our model organism is the freshwater crustacean Daphnia magna and previous work has mainly focused on its interaction with Pasteuria ramosa, a naturally occurring bacterial parasite. Recently, our efforts have turned to uncovering a viral parasite that may infect Daphnia populations in the wild. Although there are no reports of a Daphnia virus, their susceptibility to a wide range of parasites and the ecological ubiquity of viruses leads us to believe that such a parasite must exist in natural populations. Uncovering such a virus, following its dynamics in natural populations, and exploiting it in such a way that allows the establishment of a versatile experimental system are major goals. Efforts to identify and characterise a virus are currently underway. Naturally, only viruses with a clear infection phenotype are desirable and assays to quantify their detrimental effect on the host need to be developed. Finally, the desire to carry out experimental evolution and infection experiments requires that naturally caught viruses be readily cultured. This poster highlights these steps and outlines some possible methodological procedures.

Giant lysogenic viruses infecting marine macroalgae of the order Ectocarpales

Weynberg, K.D.[1,2], Bellas, C. [1], Hall, M. [1], & Schroeder, D.C. [1]
[1]Marine Biological Association of the UK, The Laboratory, Citadel Hill, Plymouth PL1 2PB
[2]Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth PL1 3DH

With the retirement of two eminent algal virologists, Prof. Dieter Müller & Prof. Russell Meints, the characterisation of the viruses that infect the different ectocarpoids has all but ceased. The ectocarpoids all fall within the order Ectocarpales, which currently includes 5 families, namely Ectocarpaceae, Scytosiphonaceae, Chordariaceae, Adenocystaceae and Acinetosporaceae. Viruses are known to infect the members within three families of the Ectocarpales – Ectocarpaceae, Chordariaceae and Acinetosporaceae. *Ectocarpus siliculosus* virus (EsV-1), *Feldmannia* sp.virus (FsV) and *Feldmannia irregularis* virus (FirrV-1) are the only members that have been studied in detail. Moreover, the sequencing of EsV-1 and more recently it's host, *Ectocarpus siliculosus*, has and will continue to dominate the landscape of ectocarpoid virology. Currently, at least five other species of ectocarpoids are known to be infected by similar viruses. Most of these viruses have only undergone a basic form of characterisation and the relationship between all these ectocarpoid viruses has yet to be determined. Here we present some preliminary genotypic data elucidating this relationship.

Personal profiles

Workshop experts

Corina Brussaard

Dept. Biological Oceanography
Royal Netherlands Institute for Sea Research
PO Box 59
NL-1790 AB Den Burg, Texel
The Netherlands

email: corina.brussaard@nioz.nl

Key research interest.

Presently working at the Royal Netherlands Institute for Sea Research, Department Biological Oceanography. Main research topics are virus ecology and phytoplankton cell mortality. Related to viral ecology, my research interests are focused on understanding and quantifying the significance of viruses as mortality agents of microorganisms (phytoplankton) and their effects on planktonic marine ecosystems and biogeochemical cycles. There are several key areas, which will give us much better insight into the role and the quantitative importance of viruses in marine ecosystems. Foremost is quantifying the ecological impact and relevance of viruses and viral mediated cell lysis on aquatic food webs and biogeochemical cycles. A second area that is still poorly understood is the role of viruses in controlling population dynamics and community structure. A third area of fundamental importance that has my interest is understanding the importance of host range and resistance to virus infection. For optimal understanding and insight, the research is performed in the field, mesocosms and laboratory.

Nicky Burbidge

Barloworld Scientific Ltd
Beacon Road
Stone
Staffordshire, UK
ST15 0SA
Tel. 01785 812121
Email: nicky.burbidge@barloworld-scientific.com

Since 1995 I have been involved in quantitative PCR which progressed from competitive PCR to real-time PCR in 1997. The main focus of my research was the use of real-time PCR and microarrays to find new therapeutic drug targets for human respiratory diseases such as cystic fibrosis, COPD and emphysema. In 2003 I joined Barloworld Scientific Techne as the Applications Specialist for real-time PCR. Based in the UK I provide applicational and technical assistance in real-time PCR for all our customers worldwide.

Jim van Etten

Department of Plant Pathology
406 Plant Sciences Hall
University of Nebraska
Lincoln, NE 68583-0722 USA
tel: 402-472-3168
fax: 402-472-2853
jvanetten@unlnotes.unl.edu
http://www.ianr.unl.edu/plantpath/facilities/Virology

I grew up in Peoria, Illinois, which is in the Midwestern part of the United States. After obtaining my PhD in Plant Pathology at the University of Illinois in 1965, I spent one year on a National Science Foundation fellowship in the Department of Genetics at the University of Pavia in Italy where I was first introduced to molecular biology. I joined the faculty at the University of Nebraska in the Department of Plant Pathology in 1966 and I have remained in Lincoln the past 40 years. My early research at Nebraska focused on two topics - molecular events associated with fungal spore germination and the characterization of an unusual virus, called phi 6 that infected a plant pathogenic bacterium.

For the past 25 years my lab has focused on the isolation and characterization of large viruses that infect algae. These viruses exist in fresh water from all over the world with natural titers as high as 100,000 infectious particles per ml. The algal viruses are among the largest viruses discovered, containing up to 400 protein-encoding genes. The biological functions of about 40% of the virus-encoded proteins are known. Many of these proteins are unexpected and have not been found in viruses previously. The algal viruses also contain other properties that are more characteristic of cellular organisms including introns and inteins. Accumulating evidence suggests these viruses have a long evolutionary history, possibly dating back to more than 2 billion years. We have a wide range of projects dealing with these viruses, ranging from basic molecular biology to ecological studies.

Keizo Nagasaki

Harmful Algae Control Section,
National Research Institute of Fisheries
and Environment of Inland Sea,
2-17-5 Maruishi, Hatsukaichi,
Hiroshima 739-0452,
JAPAN

email address: nagasaki@affrc.go.jp
http://www.nnf.affrc.go.jp/HABD/HACS/english/engmain.html

Key research interests:
- Viral impacts on harmful algal blooms
- Red tide disintegration mechanism
- Diversity of host algae and their viruses

Electron Microscopy
Electron microscopy is a useful tool to examine not only viruses' morphology but also their intracellular propagation process. In the present section, two principal methods in transmission electron microscopy will be summarized: (1) a negative staining technique using heavy metal salts to enhance the contrast between the background and the virion's image; (2) an ultra-thin section microscopy technique that provides details of the interior of the virus-infected cells. The former method is very simple and direct; in contrast, the latter analysis requires a number of troublesome procedures accompanied with use of dangerous reagents. Nevertheless, both methods are essential in studying any viruses, and I believe it is worthy for you to acquire them. Practice makes perfect. The most important thing is to try the experiments for yourself under an appropriate instruction.

John H. Paul

Biological Oceanography
Distinguished University Professor, Ph.D.,
University of Miami, 1980
Contact:
University of South Florida, College of Marine Science
140 7th Ave. South
St Petersburg, FL 33701
(727) 553-1130
jpaul@marine.usf.edu
http://www.marine.usf.edu/faculty/john-paul.shtml

The common research theme in my lab is the measurement of gene expression as a means to understanding microbially-mediated processes in the oceans. This is divided into specific areas of research that include lysogeny, phytoplankton carbon fixation, and development of sensors. Lysogeny is the process whereby a virus establishes a stable symbiosis in its host. We are examining the genomes of temperate marine bacteriophages to understand the control of lysogeny in heterotrophic bacteria and picocyanobacteria in the marine environment. Our studies in carbon fixation have focused on the control of this process in oceanic river plumes. Such plumes have tremendous CO 2 drawdown, yet also behave as areas of high levels of recycled production. We are using our experience in measuring mRNA as a surrogate for microbial gene expression in the design of hand-held and autonomous sensors (in conjunction with the Center for Ocean Technology) for the detection of noxious microorganisms in coastal environments.

Selected Publications

Patterson, S.S., E. T. Casper, L. Garcia-Rubio, M. C. Smith and J. H. Paul. 2005. Increased Precision of Microbial RNA Quantification using NASBA with an Internal Control (IC-NASBA). J. Microb. Methods (in press)

Casper , E.T., S. S. Patterson, M.C. Smith, and J.H. Paul. 2004. Development and Evaluation of a Method to Detect and Quantify Enteroviruses Using NASBA and Internal Control RNA (IC-NASBA). J. Virol. Meth. (in press)

Wawrik, B., J.H. Paul, D. A. Bronk, D. John, M. Gray. 2004. High rates of ammonium recycling drive phytoplankton productivity in the offshore Mississippi River Plume. Aquatic Microbial Ecology 35:175-184.

Casper, E.T., J.H. Paul, M.C. Smith, and M. Gray. 2004. The detection and quantification of the red tide Dinoflagellate *Karenia brevis* by real-time NABSA. Appl. Environ. Microbiol. 70:4727-4732

L. McDaniel, L. Houchin, S. Williamson and J.H. Paul. 2002. Lysogeny in Natural Populations of Marine *Synechococcus.* Nature 415:496

Griffin, D.W., Donaldson, K.A., Paul, J.H., Rose, J.B. (2003) Pathogenic human viruses in Coastal waters. Clinical Microbiology Reviews Jan 2003 p. 129-143

Ruth-Anne Sandaa

Department of Biology
Research group: Microbial ecology
University of Bergen
Post box 7800
5020 Bergen
Norway
e-mail: Ruth.Sandaa@bio.uib.no

Research experience in marine microbial ecology focusing on microbial population dynamics and biodiversity. Particularly interested in the interaction/links between bacterial and viral diversity and how it changes under different environmental conditions. The studies are conducted applying molecular methods as DGGE, PFGE, sequencing, probing and cloning.

Dr Declan Schroeder (co-organiser)

The Marine Biological Association of the UK
Citadel Hill
Plymouth PL1 2PB
UK
(tel) +44 (0)1752 633356
(fax) +44 (0)1752 633102
(web) www.mba.ac.uk

Dr Declan Schroeder is a Molecular Biologist at the MBA where he has worked since 2001. His research interests revolve around three main areas: Pathology, Genomics and Biotechnology.

Pathology: His research focuses primarily on determining the impact of viruses on biogeochemical and ecological processes. He is an associate member of the Working Group to *Investigate the Role of Viruses in Marine Ecosystems* - Scientific Committee on Oceanic Research (SCOR).

Genomics: Another key area of his research involves genome annotation and developing genomic technologies such as DNA microarrays for model marine organisms to study key biological processes. In addition, he has developed a suite of novel molecular tools to investigate how plankton diversity has changed over space and time in our oceans.

Biotechnology: In addition to addressing fundamental research questions, these virus dynamics are currently being investigated as possible novel gene delivery systems.

Curtis Suttle,
Associate Dean, Research & Academic Services
EOS, Oceanography
Univ of British Columbia
6270 University Blvd.
Vancouver, BC, V6T 1Z4
CANADA
csuttle@eos.ubc.ca, http://www.ocgy.ubc.ca/~suttle/

Curtis Suttle completed BSc (hons) and PhD degrees in Zoology and Botany, respectively, at UBC before moving in 1987 to the State University of New York at Stony Brook as the Coastal Marine Scholar at the Marine Sciences Research Institute. After 18 months he moved to the Marine Sciences Institute at the University of Texas at Austin where he was an Associate Professor. In 1996 he returned to UBC where he is currently Professor of Earth & Ocean Sciences, Microbiology & Immunology, and Botany, as well as, Associate Dean of Science for Research.

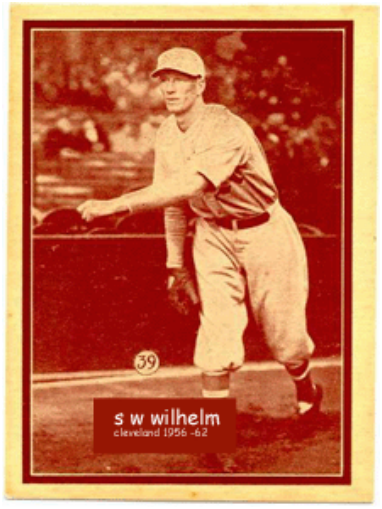
His active research program focuses on marine microbiology and includes approximately undergraduate, graduate and post-doctoral researchers. Ongoing research projects include the role of viruses in Arctic Ocean ecosystems, natural reservoirs of viruses that infect Pacific Salmon, the use of viruses as environmental proxies, the isolation and characterization of unusual viruses from the sea, and the evolution and diversity of viruses and viral communities

He has authored over 100 scientific papers and is a frequent invited speaker at Universities and International symposia. He is an active member of several scientific societies including the American Society of Microbiology (ASM) and the American Society of Limnology and Oceanography (ASLO). He has held elected office in ASM and ASLO, and has had editorial responsibilities for *Limnology and Oceanography*, *Aquatic Microbial Ecology*, *Environmental Microbiology* and *Microbial Ecology*.

Markus Weinbauer
Laboratoire d'Océanographie
de Villefranche-sur-mer (LOV)
Microbial Ecology and Biogeochemistry Group
CNRS-UPMC, UMR 7093
BP 28 (for deliveries: Chemin du Lazaret 181)
06234 Villefranche-sur-mer,
France
Email: wein@obs-vlfr.fr or Markus.Weinbauer@obs-vlfr.fr

My research interests are to quantify viral production and assess its role for controlling prokaryotic production and diversity and their link to biogeochemical cycling. I am particularly interested in comparing viral effects on ecosystem functioning and diversity to those of grazing by flagellates. The systems I am interested in range from coastal to offshore waters and the deep sea but also include freshwater environments. I also work on the co-variation between viral and host diversity and on the effects of aerosols on viral proliferation in the ocean. Recently, I became interested in the alleochemical effects of corals and benthic algae on phage-prokaryote interactions.

Steven W. Wilhelm



Department of Microbiology
The University of Tennessee
Knoxville, TN 37996
wilhelm@utk.edu

Associate Professor of Microbiology.

Also *Associate Professor of Ecology and Evolutionary Biology as well as Genome Science and Technology.*

http://web.bio.utk.edu/wilhelm/

Interests:
Research interests in the lab concern the interactions between microorganisms and biogeochemical cycles. Along with work on toxic cyanobacteria and examinations of Fe as a growth limiting agents in marine systems, the Wilhelm lab is interested in the impacts of system geochemistry on virus-mediated processes, and in turn how virus activity influences the cycling of carbon, nutrients and trace elements in aquatic systems. Questions concerning virus production rates and virus diversity top the areas of study.

Selected recent papers on virus ecology

Wilhelm SW, MJ Carberry, ML Eldridge, L Poorvin, MA Saxton and MA Doblin. 2006. Marine and freshwater cyanophages in a Laurentian Great Lake – evidence from infectivity assays and molecular analyses of *g20* genes in Lake Erie. Applied and Environmental Microbiology 72:000.

Mioni CE, L Poorvin and SW. Wilhelm. 2005. Virus and siderophore-mediated transfer of available Fe between heterotrophic bacteria: characterization using a Fe-specific bioreporters. Aquatic Microbial Ecology 41:233-245.

Strzepek, RF, MT Maldonado, JL Higgins, J Hall, SW Wilhelm, K Safi, and PW Boyd. 2005. Spinning the ferrous wheel - the importance of the microbial community in an Fe budget. Global Biogeochemical Cycles 19, GB4S26, doi:10.1029/2005GB002490,

Gobler CJ, S Deonarine, JA Leigh-Bell, MD Gastrich, OR Anderson, and SW Wilhelm. 2004. Ecology of phytoplankton communities dominated by *Aureococcus anophagefferens*: The role of viruses, nutrients, and microzooplankton grazing Harmful Algae – 3: 471 - 483.

Poorvin L, JM Rinta-Kanto, DA Hutchins and SW Wilhelm. 2004. Viral lysis as a major source of bioavailable iron in marine ecosystems. Limnology and Oceanography 49: 1734-1741.

Gastrich MD, JA Leigh-Bell, CJ Gobler, OR Anderson, SW Wilhelm and Martha Bryan. 2004. Viruses as potential regulators of regional brown tide blooms caused by the alga, *Aureococcus anophagefferens.* Estuaries 27: 112-119.

Willie Wilson (co-organiser)

Plymouth Marine Laboratory,
Prospect Place,
Plymouth,
PL1 3DH, UK
Phone +44 1752 633100
Email: whw@pml.ac.uk

Willie Wilson studied Marine Biology and Biochemistry at the University of Wales, Bangor (1987-1990) before conducting his MSc (by research) on marine cyanobacteria genetics and PhD on viruses (phage) that infect marine cyanobacteria (1990-1994). He continued for a further 4 years developing molecular tools to study marine viruses during postdoc positions at the University of Warwick. He was appointed as a Marine Biological Association Research Fellow in 1998 where he worked for 6 years setting up a group looking ant various aspects of aquatic virus molecular ecology. He took a joint appointment with Plymouth Marine Laboratory (PML) in 2002, switching to a full time post with PML in 2004 and currently manages their molecular biology facility. He will be moving to the Bigelow Laboratory for Ocean Sciences, Maine, USA in January 2007 to take up a Senior Research Scientist position.

His research focus at PML includes virus environmental genomics, virus biogeochemistry and coral viruses. He also works with PML Applications Ltd. on exploitation of virus enzymes and technology transfer initiatives.

External responsibilities include: co-founder and director of the UK Virus Ecology Group (VEG) (http://viruses.bluemicrobe.com); council member of the Challenger Society for Marine Science (CSMS); full member of the Scientific Committee on Oceanic Research (SCOR) working group on the role of viruses in marine ecosystems; chair of the *Phycodnaviridae* sub-committee of the International Committee on Taxonomy of Viruses (ICTV). Willie is also a member of the MBA of the UK; Society for General Microbiology (SGM); American Society for Limnology and Oceanography (ASLO); American Society for Microbiology (ASM).

K. Eric Wommack, Ph.D.

University of Delaware
Delaware Biotechnology Institute
15 Innovation Way
Newark, DE 19711
wommack@dbi.udel.edu
http://www.dbi.udel.edu/MOVE/MO_index.htm

I probably knew from before I could walk that I wanted to be a marine scientist. I was born under the astrological sign of Pisces, the fish. According to my mother, as an infant I crawled into the neighborhood pool, sank to the bottom for a few terrifying seconds and came out smiling and happy. At the age of nine I was disappointed to learn that I had to wait six long years before I could be come a certified diver. But, without a doubt, the life event that cemented my interest in marine science was my first visit to a Bahamian coral reef at the age of 15. Each summer for the succeeding seven years, I worked in the Florida Keys at a national Boy Scouts of America high adventure camp, known at the Florida Sea Base.

After finishing my undergraduate studies at Emory University, I never looked back at life in the burgeoning city of Atlanta, but headed off to the University St. Andrews, Scotland through the generous support of a Bobby Jones scholarship. As the official home golf and the Gatty Marine lab, the St. Andrews offered me rich life experiences that I cherish to this day. Under the advisement of Dr. Ian Johnson, I completed an M. Sc. thesis studying the relationship between muscle proteins and temperature adaptations in fish. While I enjoyed my studies on the muscle physiology of fish, I knew that I wanted to pursue field-based research on the diversity and ecology of marine microorganisms.

This desire brought me to the laboratory of the world-renowned marine microbiologist, Rita R. Colwell at the University of Maryland. Just before coming to Dr. Colwell's lab in the newly formed Center of Marine Biotechnology (COMB), the seminal 1989 paper by Bergh et al. reporting the astounding abundance of viruses in marine environments appeared in the journal, Nature. This discovery drove my dissertation research and to this day, I continue to be fascinated by the astounding abundance, diversity, and ecological impact of viruses and viral infection within the microbial communities that sustain the biosphere.

After finishing my dissertation research, I branched out from marine science into the terrestrial realm of soil microbial ecology. Through the support of a National Research Council Post-Doctoral Fellowship and under the mentorship of David Lewis and Wayne Garrison, at the US EPA; and Robert Hodson, at the University of Georgia I was involved in a project to characterize soil bacteria capable of degrading a chiral pesticide. In January, 2001 I joined the faculty of the University of Delaware through joint appointments in the Department of Plant and Soil Sciences and the Graduate College of Marine Studies. My collaborations with both soil and marine scientists has enabled me to cross traditional boundaries between terrestrial and marine research and expanded the scope of my work to include investigations in both aquatic (water and sediments) and soil environments.

GE Healthcare Demonstrators

Samantha Longshaw

sam.longshaw@ge.com

After my PhD which involved examining inter-strain variation in Equine Herpes virus populations I completed a three year post-doc using SRS (Synchrotron Radiation Source) to investigate cell penetration by Adenoviruses.

I joined Amersham Pharmacia Biotech in 2001 as the UK technical support specialist for protein purification and filtration. My current role is as Account manager-BioProcess responsible for helping large pharmaceutical manufactures and biotech companies develop their production and purification processes.

I also act as technical resource for other members of the team.

Vikki Ponting

Account Manager ~ BioProcess
GE Healthcare
Tel. 0845 850 5161
Mob. 07917 067 864
vikki.ponting@ge.com

Having previously worked for the Ministry of Agriculture Fisheries and Food, I joined Amersham International in 1988 and over the last 18 years have been involved with the manufacture of Radiochemicals, customer service, sales & customer support, Account Manager for Protein Separations and have recently taken on the role of Account Manager - BioProcess. The BioProcess role focuses on working with customers to develop purification processes for the manufacture of approved therapeutic products. This involves techniques such as chromatography, filtration, cell separation, industrial molecular biology and validation/regulatory support.
Vikki Ponting

Tanya Hayes

tanya.hayes@ge.com

I joined "The Radiochemical Centre" in 1979 as Account Manager for our German Subsidiary Company – Amersham Buchler. I moved into Freight Liaison in 1989 addressing all inadequate service issues and liaising with all departments to improve performance, as well as troubleshooting all delivery and service problems. I left what was then Amersham International in 1992. Like the proverbial bad penny I returned to Amersham Pharmacia in 2000 as Customer Service Representative! In this role I was responsible for the Proteomics and Chromatography equipment orders and administration, and also worked as Vikki's backup for Bioprocess Sales Support. I joined the Protein Separations Team as an Account Manager in April 2005 and due to the recent reorganization am now an Account Manager for Life Sciences, (GE Healthcare) focusing on start up companies and supporting our smaller accounts for all radiochemical, proteomics and Chromatography consumables.

Guest speakers

John Burden.

Pathogen Population Ecology
Centre for Ecology and Hydrology,
Mansfield Road,
Oxford. OX1 3SR
UK

01865 281630
jbur@ceh.ac.uk

The molecular ecology of viruses, particularly the interactions of baculoviruses with their insect hosts. In recent years this has focussed on persistent baculovirus infections in the Cabbage Moth and other species, looking at the distribution and maintenance of these infections at a population and individual level, and how these infections may have co-evolved with their host. Other work has focussed on the within host growth and of baculoviruses, how genetically modified baculoviruses compete with wild-type viruses as mixed infections within a single host and on developing phage based methods for the control of spoilage bacteria in metalworking fluids.

Nick Mann

Professor Nicholas H. Mann
Department of Biological Sciences
University of Warwick
Coventry CV4 7AL
UK
Tel: +44 (0)2476 523526
Fax +44 (0)2476 523701
Email: N.H.Mann@warwick.ac.uk

http://www.bio.warwick.ac.uk/res/frame.asp?ID=23

Research interests: I started my research career working on the molecular biology and biochemistry of cyanobacteria and thus it was natural to extend this interest to the marine Synechococcus strains shortly after their discovery in 1979. Very shortly afterwards the research on their phages started and the first publication came out in 1983. Most of my current interest focuses on interactions between phage and host during the infection process and also interactions between viral and grazing mortality. I am also involved in a phage therapy company.

Workshop demonstrators

Mike Allen

Plymouth Marine Laboratory
Prospect Place
The Hoe
Plymouth PL1 3DH
UK

Tel. +44 (0)1752 633100
mija@pml.ac.uk

I am based at Plymouth Marine Laboratory, where I work as a molecular biologist. My current work focuses on characterising the *Coccolithoviridae* (a family of viruses that infect *Emiliania huxleyi*) by using microarray based techniques.

Andrea Baker

Marine Biological Association of the U.K.
Citadel Hill
Plymouth PL1 2PB
UK

Tel: +44 (0)1752 633220
Fax: +44 (0)1725 633102
Email: ancba@mba.ac.uk

I am a postdoctoral research assistant currently working at the Marine Biological Association. My main research interests have been directed towards the study of freshwater cyanophages. I am now working on RNA viruses that infect the european honeybee.

Peter Bond

Scientific Officer
Plymouth Electron Microscopy Centre
University of Plymouth
Drake Circus, Plymouth
UK PL4 8AA
Tel: 44 (0)1752 233092
pbond@plymouth.ac.uk/emc
http://www.plymouth.ac.uk/pages/view.asp?page=12257

Main research interests are based on the effects of metal toxicity on the growth and ultrastructure of marine algae, but the EM Centre has links with many and varied research groups both within the university and with local collaborating institutes and research centres.

Clifford H. Thorp, Fran M. Sewell and Peter R. Bond. 1991. A self-cleaning mechanism in the operculum of *Serpula vermicularis* L. (Polychaeta: Serpulidae). Bulletin of Marine Science, Miami, 48 (2). 412-419.

Peter Bond, Maria Donkin and Roy Moate. 1997 The Development and Evaluation of Freeze-Fracture/Cytoplasmic Maceration for the Scanning Electron Microscope to Investigate Algal Ultrastructure. Micron, 28 (6). 433-438.

Peter R. Bond, Murray T. Brown, Roy M. Moate, Martha Gledhill, Stephen J. Hill and Malcolm Nimmo. 1999. Arrested Development in *Fucus spiralis* (Phaeophyceae) Germlings Exposed to Copper: A Preliminary Morphological and Ultrastructural Investigation. European Journal of Phycology, 34: 513-521.

Claire Evans

Plymouth Marine Laboratory
Plymouth Marine Laboratory
Prospect Place
Plymouth PL1 3DH
UK
Tel: +44 (0)1752 633100
Email:ceva@pml.ac.uk

I am interested in the role of viruses within the marine microbial food web and their subsequent influence on the biogeochemistry of the oceans with particular reference to the cycling of C, N, P and S.

Claudine Falla

Marine Biological Association of the U.K.
Citadel Hill
Plymouth PL1 2PB
UK
mres28@MBA.ac.uk

After completing a degree in Marine Biology and Coastal Ecology I decided to broaden my skills and focus my masters project on microbiology, with particular interest in the rapidly expanding field of aquatic virology. This has lead to the combination of my interests with a project focusing on the phenotypic and genotypic characterisation of a new marine virus infecting an Ectocarpales species.

Matt Hall

Marine Biological Association
Citadel Hill
Plymouth PL1 2PB
UK
Tel. 01752 633218
Fax 01752 633102
m.hall@mba.ac.uk

Research technician working on marine algal viruses (*Ectocarpus*, cyanobacteria and *Emiliania huxleyi*), bee viruses and characterisation of diatom nutrient transporters.

Ellie Harrison

Plymouth Marine Laboratory
Prospect Place
Plymouth PL1 3DH
UK
Tel: +44 (0)1752 633100
elha@pml.ac.uk

I am a PhD student registered at the University of Warwick. I am working with cyanobacteria *Prochlorococcus* and its co-occurring viruses. I am developing methods to determine the diversity of *Prochlorococcus* and their viruses from environmental samples.

Susan Kimmance

Plymouth Marine Laboratory
Prospect Place
Plymouth PL1 3DH
UK
Tel: +44 (0)1752 633100

sukim@pml.ac.uk

I am based at Plymouth Marine Laboratory where I am employed as a microbial ecologist. My current research interests are determining the impact of viral versus grazing mortality of phytoplankton and assessing the impact of viral infection on host photophysiology and primary production.

Jayme Lohr

Plymouth Marine Laboratory
Prospect Place
Plymouth
United Kingdom
PL1 3DH
Tel: +44 (0)1752 633 100
Email: jaylo@pml.ac.uk

Isolation and characterization of latent viruses of marine algae and coral zooxanthellae.

Steven Ripley

The Marine Biological Association of the UK,
Citadel Hill, Plymouth,
PL1 2PB, UK
Tel (office): 01752 63 3341
Tel (lab): 01752 63 3220
Tel (mobile): 07958 566 432

Email: mres@MBA.ac.uk

Areas of interest:
The study of genetic changes in *Emiliania huxleyi* blooms, associated viruses and other associated organisms over time in the North East Atlantic.

Karen Weynberg

Plymouth Marine Laboratory
Prospect Place
Plymouth PL1 3DH
UK
Tel: +44 (0)1752 633151

E-mail: kwey@pml.ac.uk

I am in the first year of a PhD based at Plymouth Marine Laboratory under the supervision of Dr Willie Wilson.
During my PhD, I will be looking to detect, isolate and characterise novel algal viruses infecting phytoplankton, specifically microalgal eukaryotes in the pico- and nano- size ranges. The project will involve genomic analyses, with a long-term view to screen for exploitable virus-specific traits.

Workshop participants

Prof. Tom Berman
Kinneret Limnological Lab.
Israel Oceanographic and Limnological Research (IOLR)
P.O.Box 447,
4950 Migdal,
Israel
E-mail: tberman@ocean.org.il

Julia A. Boras, PhD student
INSTITUT DE CIENCIES DEL MAR, CMIMA
Passeig Maritim de la Barceloneta 37
08003 BARCELONA
SPAIN
E-mail: boras@icm.csic.es

Raffaela Cattaneo (PhD student)
Laboratoire d'Océanographie de Villefranche FRANCE
Station Zoologique – B.P. 28
06234 Villefranche sur Mer
France
E-mail: cattaneo@obs-vlfr.fr

Mr Yi-Wah Chan
Biological Sciences, University of Warwick,
Coventry CV4 7AL
West Midlands
E-mail: Y.W.Chan@warwick.ac.uk

Yi-Lung Chen
Research Center for Environmental Changes
128 Sec, 2, Academia Road Nankang
11529 Taipei
Taiwan R. O. C.
E-mail: r90241213@ntu.edu.tw

Jean-Michel Claverie
CNRS
31 Chemin Joseph Aiguier
13402 Marseille
FRANCE
E-mail: Jean-Michel.Claverie@igs.cnrs-mrs.fr

Li Deng
BC5, School of Biological Sciences
University of Bristol
Woodland Road
Clifton
Bristol, BS8 1UG
UK
E-mail: bzxld@bristol.ac.uk

Melissa Duhaime
Max Planck Institute for Marine Microbiology
MarMic 2009
23859 Bremen
E-mail: mduhaime@mpi-bremen.de

Manuela Filippini
ETH/EAWAG
Limnological centre
Seestrasse 79
Kastanienbaum,
6047 Luzern
Switzerland
E-mail: manuela.filippini@eawag.ch

Mrs. Manuela Vieira Gimenes
Universidade de São Paulo
Rua Raul Pompéia, 726 ap 163
CEP 05025-010 São Paulo
BRAZIL
E-mail: manuela_vg@hotmail.com

Dr Rene Groben
Centre for Ecology & Hydrology
Lancaster Environment Centre,
Lancaster
Lancashire LA1 4AP
E-mail: rgro@ceh.ac.uk

Miss Karin Holmfeldt
University of Kalmar
Barlastgatan 11
392 31 Kalmar
Sweden
E-mail: karin.holmfeldt@hik.se

Jens Borggaard Larsen
Department of Biology, University of Bergen
Jahnbakken 5
5020 Bergen
Norway
E-mail: jens.larsen@bio.uib.no

Dr Frank Jiggins
Ashworth Labs, West Mains Road
University of Edinburgh
Edinburgh EH3 9PW
E-mail: Francis.Jiggins@ed.ac.uk

Jessica Kegel
Alfred Wegener Institute for Polar and Marine Research
Am Handelshafen 12 (Building E)
27570 Bremerhaven, Germany
E-mail: jkegel@awi-bremerhaven.de

Youngnam Kim
GDD
National Oceanography Centre,
Southampton
European Way
Southampton, SO14 3ZH
UK
E-mail: ynk@noc.soton.ac.uk

Audrey Lartigue
IGS
CNRS UPR 2589
163 Avenue de Luminy
Case 934
Marseille
France
E-mail: audrey.lartigue@igs.cnrs-mrs.fr

Joaquin Martinez Martinez
Dept. Biological Oceanography, Royal Netherlands Institute for Sea Research
PO Box 59,
Den Burg
Texel NL-1790 AB
The Netherlands
E-mail: jmartinez@nioz.nl

Steven Mauro
Mercyhurst College
501 East 38th Street
16546 Pennsylvania
USA
E-mail: smauro@mercyhurst.edu

Sandra Mesquita
Lab.Microbiologia, FCMA
Universidade do Algarve
8005-139 Faro
Portugal
E-mail: smesquita@ualg.pt

Natsuko Nakayama
Laboratory of Soil Biology and Chemistry,
Graduate School of Bioagricultural Sciences,
Nagoya University,
Nagoya 464-8601,
Japan
E-mail: natsun999@nifty.com

Dr. António Joaquim Pereira Pagarete
Station Biologique de Roscoff
Rua Nova da Misericórdia, 450
9500 Ponta Delgada
Açores
Portugal
E-mail: apereira@science.uva.nl

Verónica Parada (PhD student)
Royal Netherlands Institute for Sea Research (Royal NIOZ)
Landsdiep 4
Den Hoorn,
1797 sz Texel
The Netherlands
E-mail: vparada@nioz.nl

Mrs. Kholood Al Rifaie
Kuwait Institute for Scientific Research
Mariculture and Fisheries Department,
P.O. Box 1638,
22017 Salimya,
Kuwait
E-mail: kholood.al.rifaie@gmail.com,
krifaie@mfd.kisr.edu.kw

Sharath Srinivasiah
University of Delaware
15 Innovation Way, Room148
19711 Newark Delaware
USA
E-mail: srinivas@dbi.udel.edu,
sharath_lenks@yahoo.com

Yuji Tomaru
National Research Institute of Fisheries and Environment of Inland Sea
Fisheries Research Agency
2-17-5 Maruishi
Hatsukaichi
739-0452 Hiroshima
Japan
E-mail: tomaruy@affrc.go.jp

Mr. Garth Traxler
Pacific Biological Station, Department of Fisheries and Oceans
3190 Hammond Bay Road
Nanaimo V9T 6N7
British Columbia, Canada
E-mail: traxlerg@dfo-mpo.gc.ca

Alexandra Valdés, (PhD student)
Marine Sciences Research Center (MSRC),
Stony Brook University
Stony Brook, NY, USA 11790
E-mail: Alexandra.valdes@gmail.com

Pedro Vale
Institute of Evolutionary Biology
Ashworth Labs, The Kings Buildings, West Mains Road
University of Edinburgh
Edinburgh EH9 3JT
E-mail: pedro.vale@ed.ac.uk

Dr Arvind Varsani
Electron Microscope Unit, University of Cape Town,
Cape Town,
7701 WP
South Africa
E-mail: arwind@science.uct.ac.za

Dr. Tamar Zohary
Kinneret Limnological Lab.
Israel Oceanographic and Limnological Research (IOLR)
P.O.Box 447,
14950 Migdal
Israel
E-mail:tamarz@ocean.org.il

EXPERT ADDED IN PROOF

Amy M. Chan
(*helping with EFM session***)**
Research Scientist
Earth & Ocean Sciences
University of BC
6270 University Blvd
Vancouver, BC V6T 1Z4
Canada
Tel: 604-822-0583
Email: achan@eos.ubc.ca