Environmental Microbiology

Microbial Source Tracking and Quantitative Detection of Potential Pathogens in Roof Harvested Rainwater

Warish Ahmed and Ted Gardner

March 2008

Brief background of recently completed project

1. The "Smoking Gun" study

Microbial source tracking (MST) methods to estimate of site impacts of on-site wastewater treatment systems in Pine Rivers Shire

Clients:

- Healthy Waterways
- SEQ Catchment
- Local councils

Collaborators:

- University of the Sunshine Coast (USC)
- Pine Rivers Shire

Brief background of ongoing project

2. PCR detection of pathogens in rainwater

Real-time PCR detection of pathogens in roof harvested rainwater samples collected from Southeast Queensland

Clients:

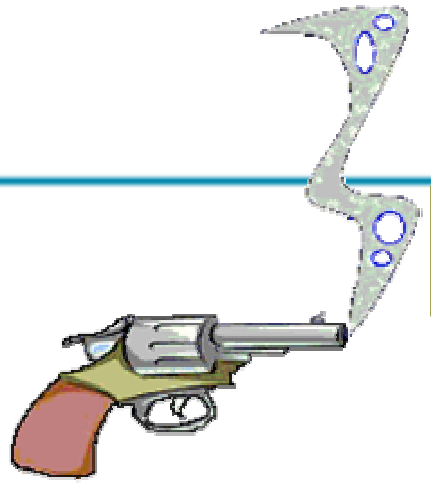
- Local Councils
- State Government
- Qld Water Commission

Queensland water Commission

Collaborator:

- Qld University of Technology

Queensland University of Technology
Brisbane Australia



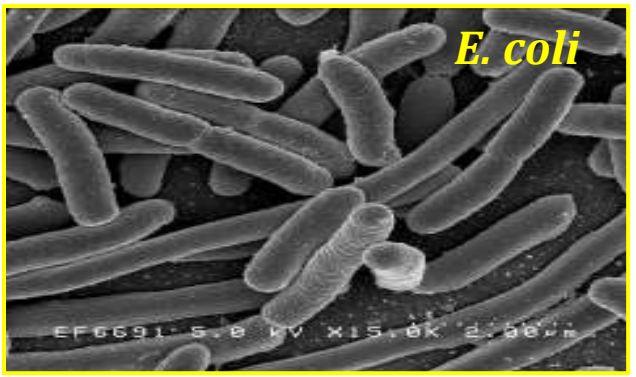
Overview of "Smoking Gun" Scoping study

Aim:

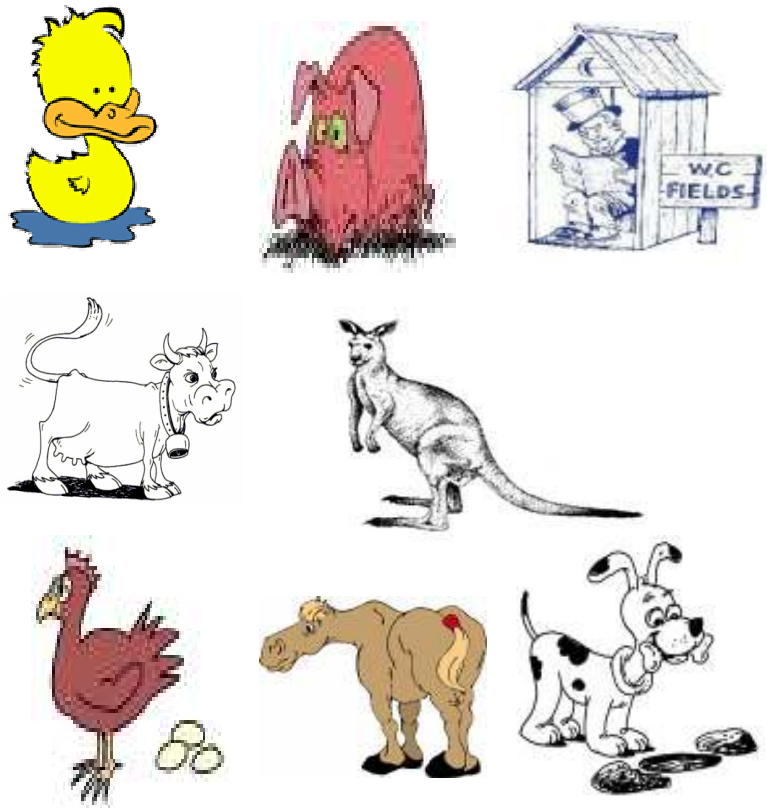
Identify *human faecal pollution* in stormwaters from non sewered catchments in Pine Rivers Shire via septic systems

What is Microbial Source Tracking (MST) ???

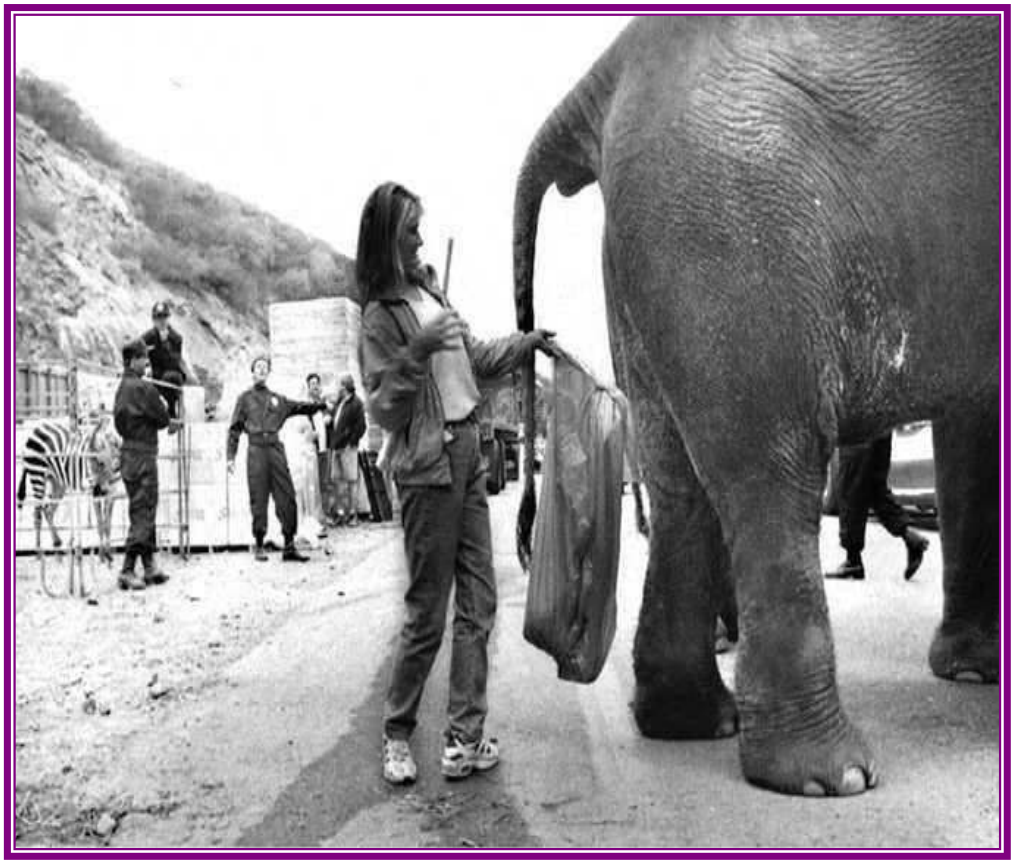
- Methods to identify the sources of faecal pollution in waters
- Fingerprints of indicator bacteria found in sources are compared to the fingerprints found in water samples
- Experimental technique gaining popularity



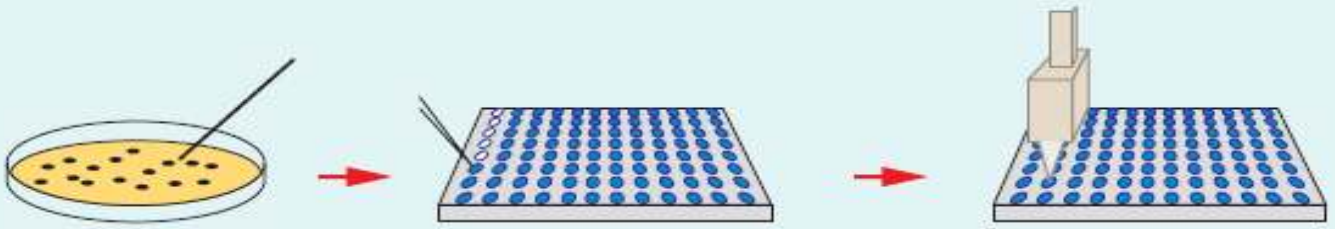
Identify the dominant sources of faecal pollution



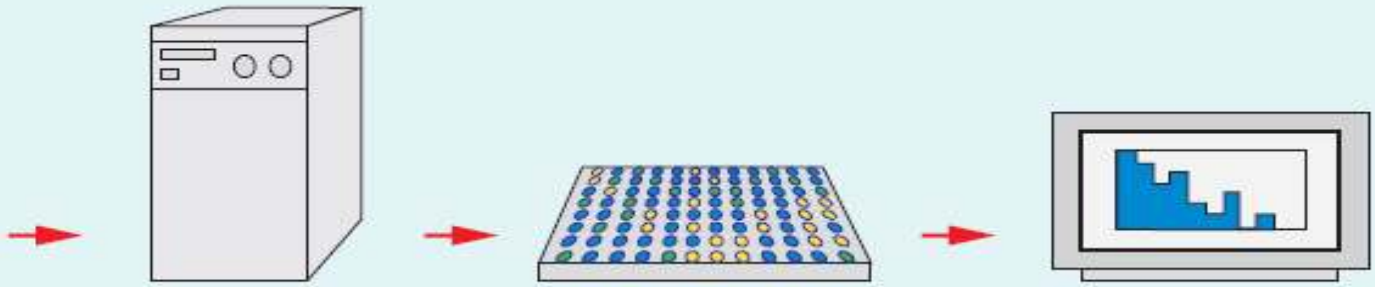
Build a faeces database



Biochemical fingerprinting procedure

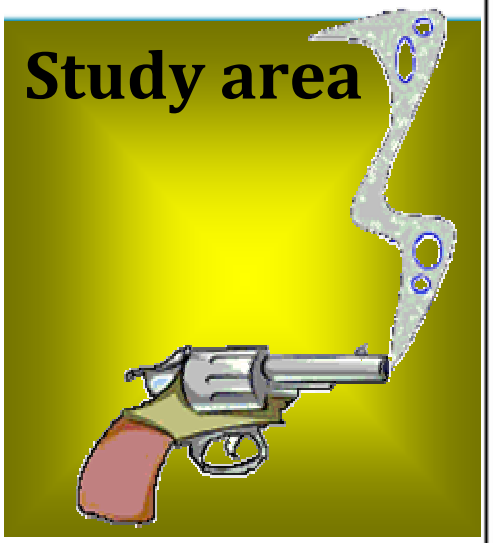


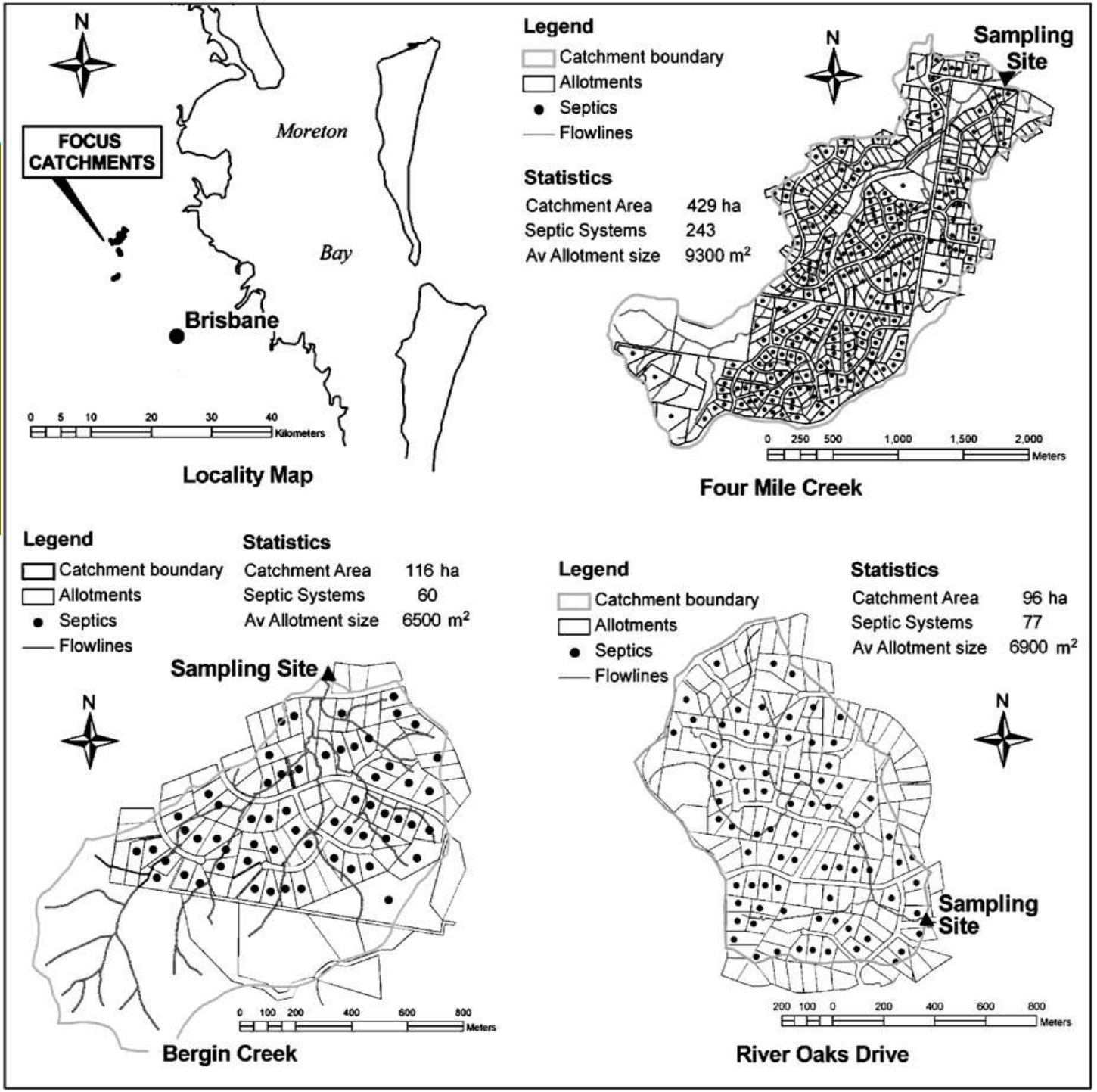
Bacterial isolates are inoculated into the PhPlates containing the growth medium and 11 discriminatory substrates



Plates are incubated and read at different time intervals; data transferred to a computer and processed

Study area

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FOCUS CATCHMENTS
Moreton
Bay
Brisbane
0 5 10 20 30 40
Kilometers
Locality Map
Legend
Catchment boundary
Allotments
Septics
Flowlines
Statistics
Catchment Area 429 ha
Septic Systems 243
Av Allotment size 9300 m²
Sampling Site
0 250 500 1,000 1,500 2,000
Meters
Four Mile Creek
Legend
Catchment boundary
Allotments
Septics
Flowlines
Statistics
Catchment Area 116 ha
Septic Systems 60
Av Allotment size 6500 m²
Sampling Site
0 100 200 400 600 800
Meters
Bergin Creek
Legend
Catchment boundary
Allotments
Septics
Flowlines
Statistics
Catchment Area 96 ha
Septic Systems 77
Av Allotment size 6900 m²
Sampling Site
200 100 0 200 400 600 800
Meters
River Oaks Drive

Catchment sampling

- Number of samples collected 21
- 19 base flow and 2 rising stage
- Up to 7 samples were collected from each site
- Rainfall 18-30 mm

River Oaks Drive

Four Mile Creek

Bergin Creek

Quantification of faecal pollution

Bergin Creek

Sources	*E. coli*	Ent
Human	8%	9%
Animal	53%	57%
Unknown ???	39%	34%

Four Mile Creek

Sources	*E. coli*	Ent
Human	4%	4%
Animal	55%	66%
Unknown ???	41%	30%

River Oaks Drive

Sources	*E. coli*	Ent
Human	10%	9%
Animal	48%	65%
Unknown ???	42%	26%

Harsh realities

Large database is required for success

At what cost?

Only Fraction of indicator can be quantified

What about unknowns?

More harsh realities

Host-specificity

E. coli and enterococci lack host-specificty!!!

Database catchment specific

Need a new database for each catchment!!!

Alternative approaches for MST

- **PCR based detection of human-specific molecular "marker"**

A molecular "marker" can be defined as a specific gene or sequence of a gene that is associated with faecal indicator of a particular host.

- **For the first time in Australia, we introduced 3 human-specific PCR markers:**

***Bacteroides* HF183**

***Bacteroides* HF134**

E. Faecium esp

Advantages

- ✓ No database is required
- ✓ Rapid
- ✓ Human-specific
- ✓ More sensitive and accurate measures of faeacl pollution
- ✓ Comparatively cheaper

Limitations

- × Markers not available for wild animals
- × Host-specificity needs to be tested before field application
- × The concentration of some of the markers could be low
- × Quantitative methods are not available for all markers

What is Polymerase Chain Reaction (PCR)

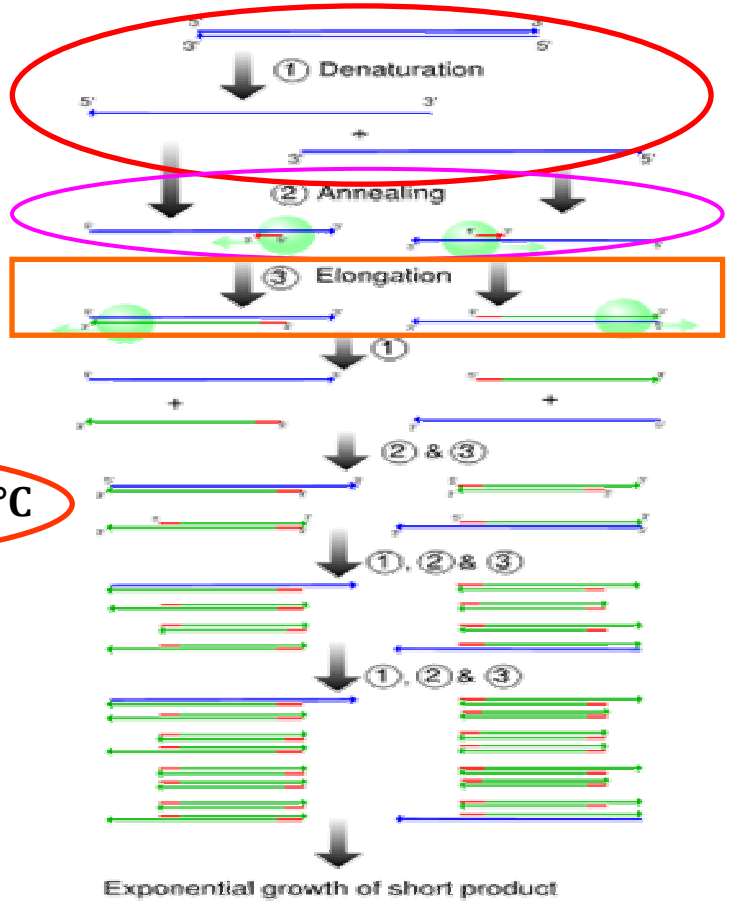
- **The PCR is a technique for copying a piece of DNA a billion-fold**
- PCR requires

1) an enzyme called *Taq*
2) Short pieces of DNA called primer
3) DNA template to copy

Double-stranded DNA separation or denaturation at 95°C

Primer annealing to template DNA at 59°

Primer elongation at 72°C



Conventional PCR

Master Mix (*Taq*, MgCl2, DNTPs)

Forward primer

Reverse primer

DNA template

Thermal Cycler (30 cycles takes about 2-3 hrs)

Gel electrophoresis

Gel documentation

How good the PCR markers are???

- 52 samples from septic and STP
- 155 samples from 12 animal species

Each faecal sample was weighted

DNA was extracted Using DNA stool kit

Samples were tested with PCR using specific primers

Host-specificity results……

Sources	HF183	HF134	*esp*
Septic system	12/12	12/12	7/12
Primary influent	15/15	15/15	15/15
Secondary effluent	15/15	15/15	14/15
Treated effluent	10/10	9/10	0/10
Ducks, chickens	0/30	0/30	0/30
Kangaroos, deer	0/25	0/25	0/25
Cattle, horses, goats	0/44	0/44	0/44
Dogs	0/20	7/20	0/20
Pigs	0/6	0/6	0/6
Pelican, wild birds	0/20	0/20	0/20
Goats, sheep	0/20	0/20	0/20
Specificity	100%	95.5%	100%

Detection of faecal pollution in Pine Rivers Catchment

Catchments	HF183	HF134	*esp*
Bergin Creek S1	+	+	+
Bergin Creek S2	+	+	+
Bergin Creek S3	+	+	-
Bergin Creek S4	-	-	+
Four Mile Creek S1	-	+	+
Four Mile Creek S2	+	+	+
Four Mile Creek S3	-	-	-
River Oaks Drive S1	-	-	+
River Oaks Drive S2	-	-	-
River Oaks Drive S3	-	-	-

Conclusions……"the smoking gun study"

- Both biochemical fingerprinting method and PCR markers indicated human faecal pollution in storm water samples collected from 3 catchments in Pine rivers Shire
- According to biochemical fingerprinting method, the percentage of human derived faecal pollution was lower than animal faecal pollution.
- Host-specific molecular markers performed well in identifying human sourced faecal pollution

QC/QA and Peer review

- For PCR analysis, peer reviewed methods were used as there is no standard method available for PCR
- PCR detection of markers were set up in consultation with the researchers who orginally developed these methods
- Each manuscripts was sent to independent reviewers in the field of MST before submission in a journal
- Each manuscript has gone through at least 7 independent reviews before being accepted for publication

Publications from "the Smoking Gun" scoping study

International peer reviewed journals

1. Ahmed *et al.* (2007) – Water Research (MST special issue)
2. Ahmed *et al.* (2008a) – Letters in Applied Microbiology
3. Ahmed *et al.* (2008b) – Journal of Environmental Quality (in press)
4. Ahmed *et al.* (2008c) – Journal of Applied Microbiology

National journal

1. Ahmed *et al.* (2008d) – AWA Water (Review article)

Exploring quantitative PCR

- Quantitative PCR also called real time PCR

- Detection and quantification of fluorescence reporter which increases in direct proportion to the amount of PCR product in a reaction

- Does not measure the end product like conventional PCR, instead its measure product in real time

Real-time PCR Cycler

Liquid Handler

Real time PCR machine

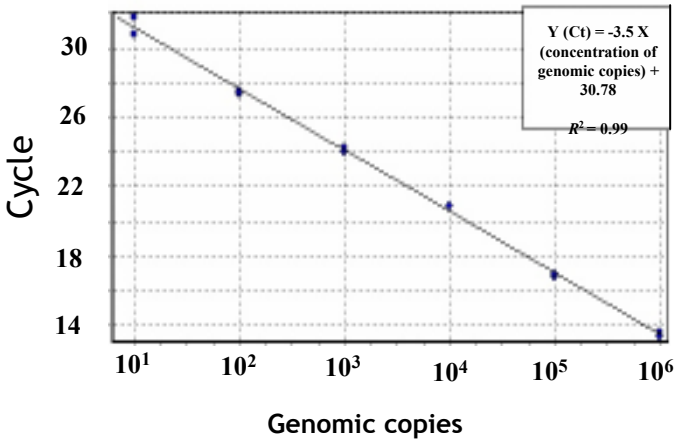
Real-time PCR Quantification process

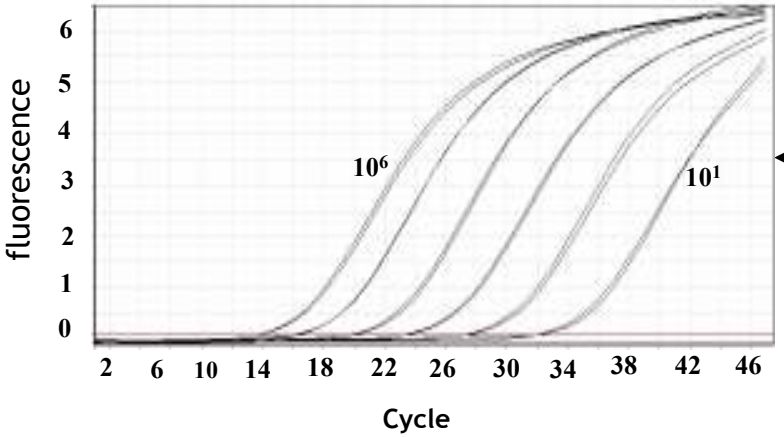
gDNA

Quantified using Spec (ng/µl)

Gene copy numbers are calculated using formula

Gene copies are serially diluted ranged from 10^7 to 10^0/µl of DNA

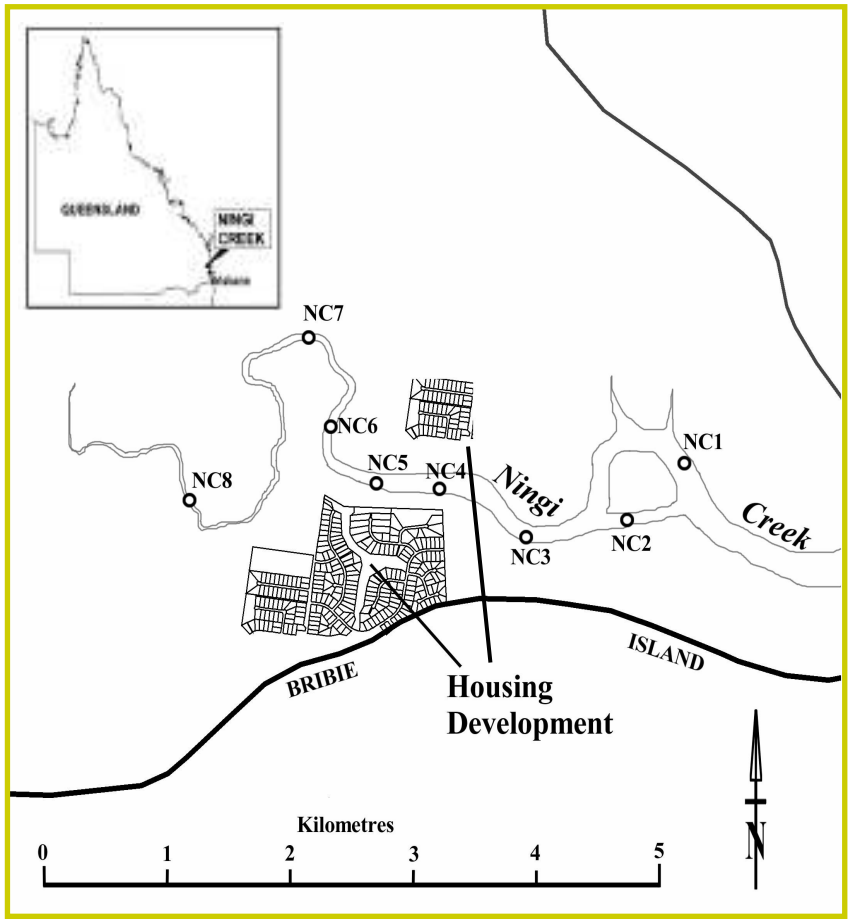




The concentration of HF183 and the *esp* marker in sewage

Raw sewage	HF183 gene copies/100 mL	*esp* gene copies/100 mL
STP 1-Sample 1	9.3×10^9	3.8×10^4
STP 1-Sample 2	3.9×10^9	1.5×10^4
STP 1-Sample 3	4.6×10^9	1.3×10^4
STP 1-Sample 4	7.3×10^9	2.3×10^4
STP 2-Sample 1	9.1×10^8	1.1×10^4
STP 2-Sample 2	2.1×10^9	2.0×10^4
STP 2-Sample 3	1.3×10^9	9.8×10^3
STP 2-Sample 4	9.8×10^8	1.0×10^4

Application of the *esp* marker in Ningi Creek catchment



Mixed landuse catchment

Entire catchment serviced by septic tanks

A recent study used ARA database and identified human sourced faecal pollution (Carroll *et al.* 2007)

16 grab samples were collected on 2 occasions after storm events (76 mm)

MST Results

Sampling sites	*esp* gene copies/100 mL
Event 1 NC1	1.1 X 10^2
Event 1 NC3	1.6 X 10^2
Event 1 NC4	5.3 X 10^2
Event 1 NC6	5.2 X 10^2
Event 2 NC4	4.3 X 10^2
Event 2 NC5	3.1 X 10^2

PROOF IN CONCEPT as the number would vary STP to STP

1.6 X 10^4 marker = 100 mL raw sewage

2.9 X 10^2 marker = 100 mL creek water

Therefore, 100 mL of creek water samples contained 1.8 ml of raw sewage

Campylobacter spp. 180 cfu
Salmonella spp. 9 cfu
Rotavirus 720 pfu
Giardia lamblia 180 cysts
Cryptosporidium parvum 0.36 oocysts
Adenoviruses 1100 genomic copies
Noroviruses 90 genomic copies

Journal Publications

International peer reviewed journals

1. Ahmed *et al.* (2008e) – Environmental Microbiology (under review)
2. Ahmed *et al.* (2008f) – Water Science and Technology (under review)

International conference

1. Health Related Water Microbiology (HRWM) – Tokyo 2007 (poster presentation)

Limitations faecal indicators

May originate from non-faecal sources

Ability to replicate in environmental waters

Cannot be used to differentiate the sources of faecal pollution

Weak association with the presence of pathogens

How about direct monitoring of pathogens ???

Direct monitoring of pathogens is an attractive option!!!

Conventional culture methods

- Injured or stressed cells
- Viable but not culturable (VBNC)
- Labour intensive
- Lack of sensitivity

PCR-based methods

- Direct monitoring of pathogens
- Detect pathogens that are difficult to grow
- Rapid
- Sensitive
- Inability to distinguish between viable and non-viable cells

Pathogens in roof harvested rainwater

Aims:

1. Detection of pathogenic microorganisms in roof harvested rainwater using PCR

2. Quantification of *Campylobacter jejuni*, *Salmonella* spp., *Legionella pneumophila*, and *Giardia Lamblia* using real-time PCR

3. Quantitative Microbial Risk Assessment (QMRA) of rainwater

Microbiological quality of rainwater

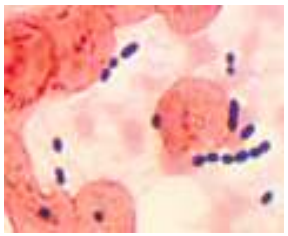
- ***Rainwater quality is generally acceptable for drinking and household use***
- ***Studies in New Zealand and in the USA reported the presence of enteric pathogens in rainwater samples***
- ***The quality of rainwater is assessed based on the concentration of E. coli***
- ***Question remains "what is the correlation between E. coli and pathogens in rainwater???***

Faecal indicators and pathogens

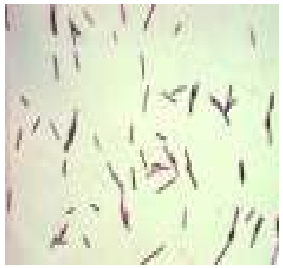
No. of samples tested = 27

Faecal indicators tested

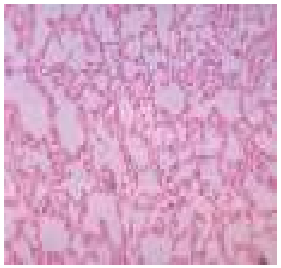
E. coli



Enterococci

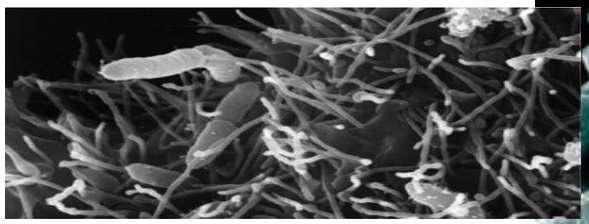


C. perfringens

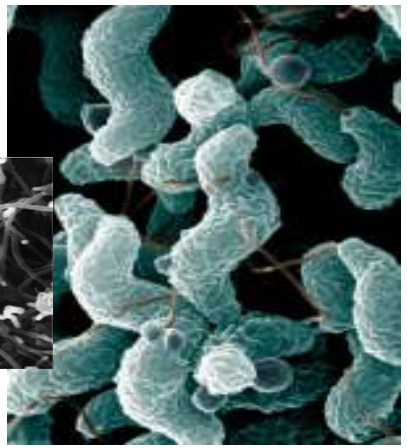


Bacteroides spp.

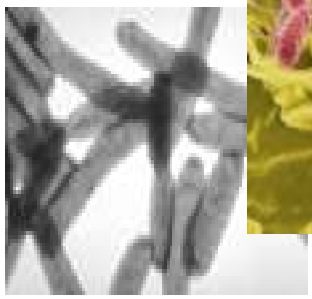
Pathogens tested



Aeromonas hydrophila



Campylobacter



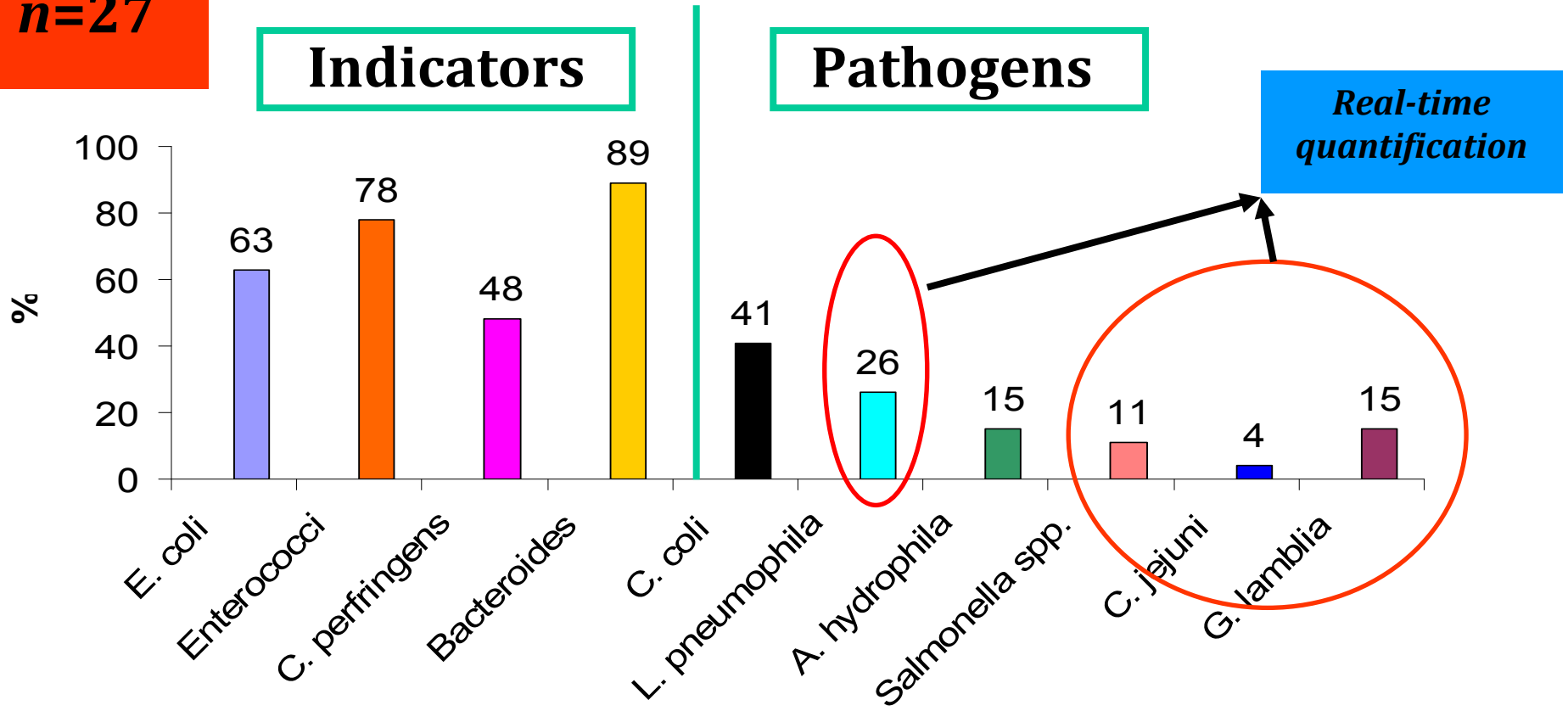
L. pneumophila

Salmonella

G. lamblia

Preliminary results……

***n*=27**



Our future.....Microbiological water quality toolbox

Indicators	MST	Pathogens
Faecal coliforms	Biochemical fingerprinting	*C. jejuni, C. coli*
E. coli	ARA	*E. coli* O157, VT1, VT2
Enterococci	MLST typing	VRE *(vanA, vanB, vanC)*
C. perfringens	Human-specific *Bacteroides*	*Salmonella spp.*
Bacteroides spp.	Human-specific *esp*	*L. pneumophila*
Coliphage	Human-specific *E. coli* clone	*A. hydrophila*
F+RNA coliphage	Cattle-specific *Bacteroides*	*G. lamblia*
	Human-specific adenovirus	*C. parvum*
	Human-specific polyomavirus	Adenoviruses
	Dog-specific *Bacteroides*	Polyomaviruses
		Noroviruses
		Rotaviruses

Peer review and publication

- *For indicator analysis standard methods were used and for PCR, Peer review methods were used*
- *The sensitivity, specificity, and intra and inter assay variability, and performance are documented for each PCR method*
- *Proficiency testing ???*

Manuscript

Ahmed *et al.* 2008g. Applied and Environmental Microbiology – has been peer reviewed by 2 independent reviewers who are not related to our work – awaiting submission

Quality of researchers

Warish Ahmed

PhD in MST (2005)
13 journal papers
6 papers are being considered for publications
Reviewed 19 Microbial water quality related research papers since 2005

Flavia Huygens

PhD in Molecular Microbiology (1992)
Experienced working with Campylobacter and MRSA
23 journal papers

National and International peer

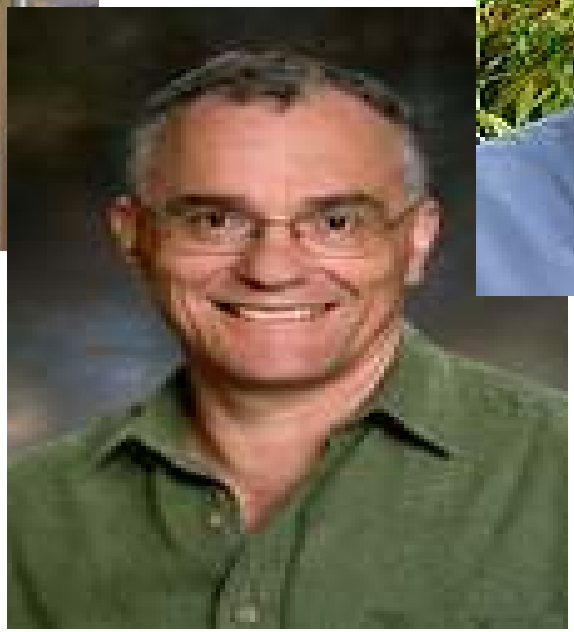
Dr. H. Katayama (Japan)

Dr. G. Reischer (Austria)

Dr. M. Katouli (Australia)

A/Prof. S. Jiang (USA)

Prof. Joan Rose (USA)



Prof. D. Gordon (Australia)

Dr. G. Hansman (Japan)