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Identifying the Biological and Geographical Origins of Faecal Contamination

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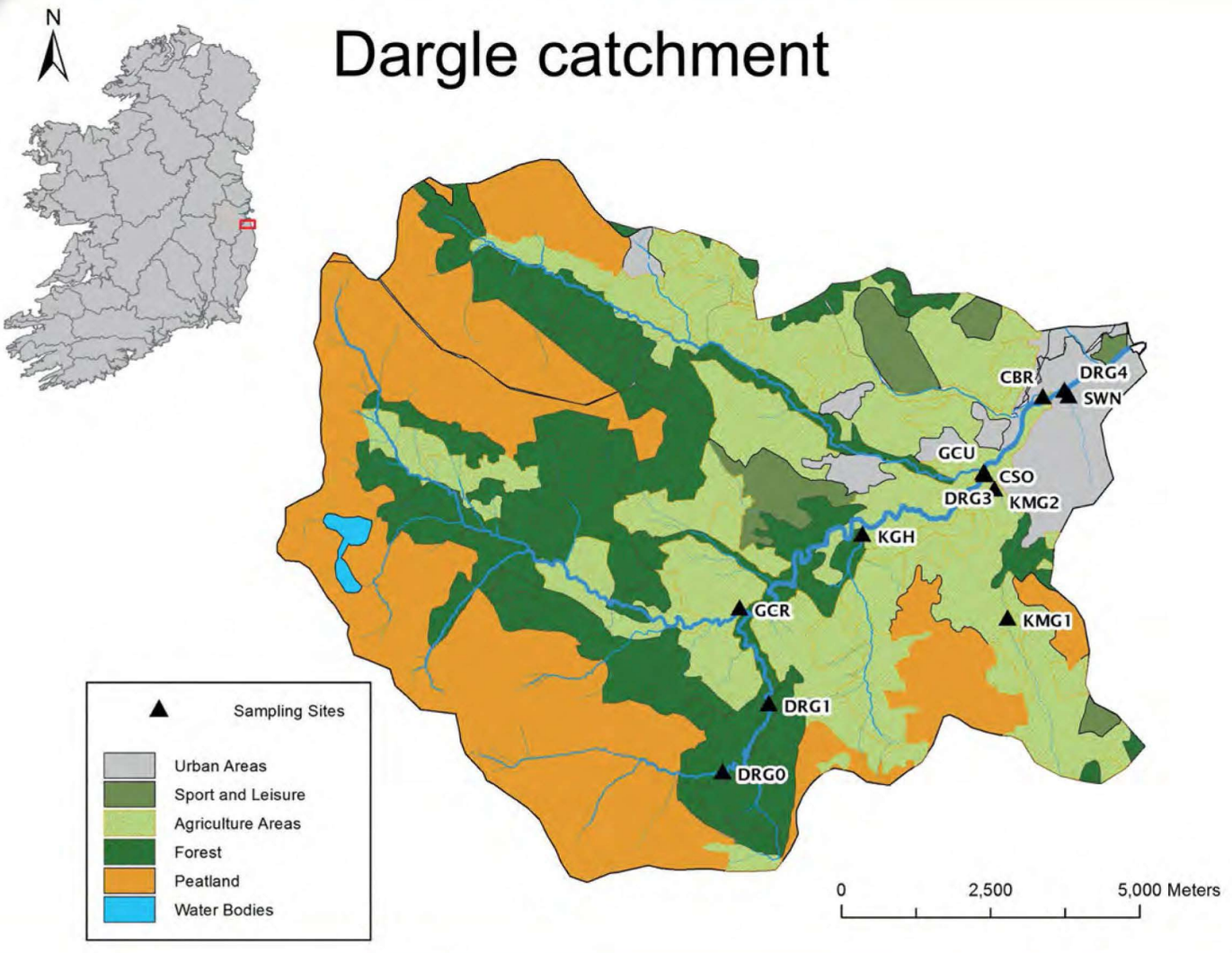

Comhshaol, Pobal agus Rialtas Áitiúil
Environment, Community and Local Government

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ENVIRONMENTAL PROTECTION AGENCY

The Environmental Protection Agency (EPA) is responsible for protecting and improving the environment as a valuable asset for the people of Ireland. We are committed to protecting people and the environment from the harmful effects of radiation and pollution.

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- the contained use and controlled release of Genetically Modified Organisms *(GMOs)*;
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- Office of Environmental Enforcement
- Office of Environmental Assessment
- Office of Radiological Protection
- Office of Communications and Corporate Services

The EPA is assisted by an Advisory Committee of twelve members who meet regularly to discuss issues of concern and provide advice to the Board.

EPA Research Programme 2014-2020

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DISCLAIMER

The EPA Research Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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Executive Summary

The aim of this project was to develop a tool box, Microbial Source Tracking (MST), to enable water quality managers to identify the biological and geographical sources of faecal pollution of water bodies. This project made use of genetic markers that are specific for a bacterial species inhabiting the intestinal tract of a particular animal or human, and of markers that are specific for mitochondrial DNA of an animal, in order to identify the biological source of faeces and faecal water pollution. The detection method is based on the polymerase chain reaction (PCR), which was used in both a qualitative (end-point PCR) and a quantitative (qPCR) manner.

Initially, MST markers specific for human, ruminant, sheep, horse, pig and gull faeces were validated using end-point PCR with a panel of faecal samples obtained from humans and animals, as well as from raw and treated sewage. This showed that the sensitivity of these MST markers was at least 73%, and the specificity was at least 87%. In addition, qPCR was used to determine the level of these markers per 100 mg of faeces, showing that the level of these markers was several orders of magnitude higher in the target species than in others.

Field testing of the MST markers was carried out in the Dargle catchment, which consists of agricultural, urban and pristine areas. Water samples taken at 12 sampling stations, as well as at the outfall of the Enniskerry wastewater treatment plant (WWTP), were analysed for the presence of faecal indicator bacteria and MST markers. MST analysis, using end-point PCR, showed a clear correlation between land use and the presence of MST markers. For example, the upstream areas of the Dargle and Glencree Rivers mainly contained the ruminant MST marker, whereas the Swan River, flowing through an urban area, mainly contained the human MST marker. Refinement of the analysis using qPCR identified three areas in the catchment that were mainly polluted by human faecal pollution: the Dargle downstream of the outfall of the Enniskerry wastewater treatment plant, and the Kilmacanogue and Swan Rivers.

The use of MST as a forensic technique to identify both the biological and geographical source of pollution was demonstrated in the Swan River, which is almost exclusively polluted by human faeces. Water samples taken along the river, in between its source and its discharge in the Dargle, were analysed by qPCR, showing that the source of human faecal pollution is located in between the Wheatfield Estate and Hollybrook Park. Based on the average flow rate and on the levels of the human MST marker in the Swan River, the authors estimated that at least 48 kg of human faecal matter per day is discharged into the Dargle from the Swan River, accounting for 3% of all human faecal matter produced in the Swan catchment.

In order to obtain insight into the behaviour of the MST markers following release into the environment, 200 L mesocosm experiments using Dargle river water were carried out that estimated the die-off rates of *E. coli,* Enterococci and the human, ruminant and horse MST markers. The T_{90} values of the MST markers ranged from 4.8 to 5.7 days. However, it was shown that the live MST markers disappeared after 24 hours following release into the mesocosm.

In summary, this project has validated eight MST markers for use in Ireland and analysed the performance of three (human, ruminant and horse) markers in great detail, as part of a study characterising the Dargle catchment. In addition, the project provided proof of concept of the application of MST as a forensic tool in identifying both the geographical and biological source of faecal pollution, as well as in estimating the amount of faecal matter that is deposited in a river.

Introduction

The Water Framework Directive (2000/60/EC) takes a holistic approach to pollution regulation on a river catchment basis. At first reading, it may seem to cater for just the ecological and the physicochemical aspects of water quality. However, the regulation of microbial quality is implicit, in that all the Community water legislation is ensconced within the Directive; for example, designated bathing waters are scheduled as Protected Areas in the Directive. The EU Commission Communication (2000, p. 4), proposing revision of the then-in-force Bathing Water Directive (EEC/76/160), verified this, stating that "the (revised) Bathing Water Directive should be the driver for a focussed implementation of the Water Framework Directive …….". Importantly, the Water Framework Directive takes a reactive approach, requiring (Article 11.5) that "where monitoring or other data indicate that (environmental quality) objectives……are unlikely to be achieved, the Member State shall ensure that: —the causes of the possible failure are investigated….". This imperative though, bears not just on designated bathing waters, but on that of the water bodies used for the extraction of potable water and for aquaculture as well.

In particular, the revised Bathing Water Directive (2006/7/EC) came into effect on the 24th of March 2006. It sets tighter standards than the previous Directive. Implementation of the new standards will result in an increase of non-compliant Irish bathing areas, if no action is taken. Key features of the revised Directive include discounting of samples and establishment of a 'bathing water profile'. Microbial water quality classification will be based on four-year[1] monitoring data using 95 or 90 percentiles. Samples may be discounted when pollution is short-term and predicted, and the cause can be identified. Article 6 of the Directive calls for the implementation of a 'bathing water profile', which calls for "identification and assessment of causes of pollution that might affect bathing waters and impair bathers' health" (Annex III, Section 1b).

A major obstacle in achieving compliance with the standards is diffuse or non-point source pollution. In contrast to pollution arising from sewage or industrial effluent, non-point source pollution emanates not from just one single location, and is therefore hard to manage. For example, pollution arising from agricultural land run-off, sewer misconnections or wild animal excrements is hard to rectify, as neither location nor identity of the contaminating factor can be immediately ascertained. It is clear, therefore, in respect to this directive, that tools for identification of the source of pollution are required in order to; manage water quality adequately to meet the required standards, allow for discounting of samples for 'short-term pollution', establish a 'bathing water profile' as required by the Directive, and identify measures to correct the problem. The methodologies used to identify the biological source of faecal pollution are known as Microbial Source Tracking (MST), which comprise a wide range of technologies and approaches.

This EPA Research Report project is based on an EU-funded project 'Improvement of Coastal and Recreational Waters' (ICREW), which was funded by the EU under INTERREG-IIIb and was

[1] Based on three-year monitoring data in special cases

completed in 2006. As part of the ICREW project, all microbial source-tracking methodologies available at the time were evaluated, resulting in the selection of two methods for extensive validation and subsequent assessment in the field in the participating countries. Criteria included; potential portability, potential for high throughput analyses, cost, ease of implementation and need for infrastructure investment.

One of the selected methods was identification of faecal pollution by PCR amplification of bacterial DNA from a host-specific bacterial species, in particular Bacteroidales species. This anaerobic bacterial genus is present in high numbers in the intestinal track of animals and humans. The group of Kate Field identified host-specific Bacteroidales species, allowing these to be used as a microbial source tracking tool (Bernhard & Field, 2000). In the ICREW project, this methodology was validated for use in the Atlantic Rim countries (it only had been tested in Oregon). The methodology proved highly successful in discriminating between ruminant (e.g., bovine) and human pollution, with high specificity and sensitivity values (>97%; n=284) (Gawler *et al.*, 2007). In Ireland, these human and ruminant markers were extensively field-tested (Bannow Bay; Dargle and Tolka Rivers), showing clear correlation between the source of faecal contamination and land use. However, the project also highlighted that significant regional differences in specificity and sensitivity exist, showing that methodologies developed elsewhere cannot be transposed to other countries without a priori local validation.

Aims of the Project

Considering the usefulness of microbial source tracking, it made sense to further expand this method, both 'horizontally' and 'vertically'. The former entailed the validation of novel markers for use in Ireland that can be used to identify additional sources of pollution, such as other farm animals and wildlife. This particular area of research has attracted considerable interest and has led to the development of markers that can be used to identify, for example, pig and horse faecal pollution. Vertical expansion of the method entailed an increased knowledge of the markers that have already been developed and validated, which will provide greater confidence in the method. In addition, it is required for a future use of the method in a quantitative manner.

Horizontal expansion of MST technology

A key area of interest is the development of additional markers to detect contamination by animals other than ruminants and humans. A number of laboratories worldwide have developed new markers that may be of use in Ireland. However, as the ICREW project convincingly demonstrated, markers need to be extensively validated and tested under local field conditions before they can be employed in Ireland (Gawler *et al.*, 2007). The aim of this part of the proposal was therefore to determine the sensitivity and specificity of these newly-developed markers for gulls, sheep, pigs and horses, for use in Ireland. The outcome of this part of the current proposal provided Ireland with a full suite of validated and characterised markers to differentiate between major sources of faecal contamination, which is critical in meeting the stringent microbiological standards set out in the revised Bathing Water Directive.

Vertical expansion of MST technology

The main aim of vertical expansion of the host-specific MST markers system was to gain a deeper understanding of the relationship between the marker and host, as well as the marker and its environment. The ultimate goal of MST is to develop a fully quantitative methodology, which aims to provide managers of water quality with percentage contribution of each source of faecal contamination. It should be realised that the current system is qualitative only, providing no data on relative contributions. As more markers become available, it is critical to have insight into the relative contributions of each source of faecal contamination, in order for managers to take the appropriate corrective action. Quantification of the marker itself is relatively straightforward using qPCR. However, this does not amount to obtaining quantitative data on the levels of faecal contamination. Additional information is required. This project aims to obtain insight in some key facets:

a) Although Bacteroidales species are abundant in faecal matter, the markers used in Microbial Source Tracking target only a small number of species within the overall Bacteroidales population. The aim of this subsection is to determine the abundance of the marker species within the overall microbial population in faeces of humans and a number of animals, as well as in sewage. The importance of this is self-evident when considering that, if a marker species is far more abundant in one host relative to the other, the impact of a perceived source of contamination on water quality may be over- or under-estimated.

b) The ICREW project demonstrated that the sensitivity of the ruminant and human MST markers is high, i.e. most individual human and bovine faecal samples carry the appropriate marker. However, no data are available regarding the stability of the level of the marker within and between hosts. This means that the level of the marker may vary, depending on the season, or different individuals harbouring different quantities of target species. This information is vital in determining the level of confidence in the system.

c) As different Bacteroidales species are targeted as markers for different hosts, they are likely to display different susceptibilities to the environment, resulting in differential die-off rates that may skew the data obtained. Information regarding the behaviour of the marker in the environment is therefore critical.

Project Structure

The project consisted of six work packages, one of which, work package 6, was added in a no-cost extension after completion of the project. Together, these work packages address all aims of the project.

Work package 1: Validation of additional MST markers for faecal contamination

As stated in the above, there may be significant variations in the specificity and sensitivity of MST markers, as was observed in the ICREW project. This work package therefore validated additional markers for use in Ireland using faecal samples from humans, as well as from a number of relevant animals.

Work package 2: Field testing of validated markers

The validated MST markers were used to analyse a well-characterised catchment (Dargle) with contrasting land use. In this work package, both qualitative (end-point PCR) and quantitative (qPCR) methods were used.

Work package 3: Levels of marker species in their hosts

The aim of this work package was to gain insight into the abundance of MST in their hosts (human and animals) in terms of gene copies (gc) per gramme of faeces. This information is essential in relating the level of a MST marker to the amount of faecal matter.

Work package 4: Quantitative analysis of contamination in the Dargle catchment

The aim of this work package was to quantify the levels of MST markers detected in the sampling of the Dargle in work package 2, using qPCR. Although this is not a quantitative determination of the level of faecal pollution from these sources, it provides insight into the fluctuation levels of the markers, which is of particular interest when comparing high and low water flow conditions.

Work package 5: Determine the differential die-off rates of microbial source tracking markers in a microcosm

Bacteroidales species are obligate anaerobic bacteria of the intestinal track, and will therefore be subjected to suboptimal conditions following their release into the catchment, resulting in their disappearance. The aim of this work package was to determine the die-off rate of the MST markers.

Work package 6: Modelling of MST markers in the Dargle catchment

This work package was added following completion of the project, as a no-cost extension. During this project, the Dargle catchment was instrumented with a number of flow gauges, rain gauges and weather stations. In addition, water samples taken during high and low flow conditions were analysed for faecal indicator bacteria. Together with hydrological data, this allowed for the development of a catchment model. The aim of this work package was to determine the effects of bad weather events on water quality.

Methods

Sample collection

Faecal samples

A total number of 181 faecal samples were analysed in this study. Individual fresh faecal samples were collected from different animals: pigs (n=20), horses (n=27), sheep (n=25), cattle (n=22), deer (n=15), goats (n=2), peacocks (n=3) and a donkey (n=1) in several farms, a veterinary hospital, and the countryside around Dublin. Samples from gulls (n=32) and swans (n=8) were collected in Bray Harbour and Ireland's Eye Island. The main gull species in these environments are the great black-backed gull (*Larus marinus*), European herring gull (*Larus argentatus*) and the common gull (*Larus canus*). Faecal samples of each animal were collected from at least two different locations. Twenty-six human faecal samples, which did not receive antibiotic treatment, were obtained from Crumlin Hospital (Dublin). Ethical permission for human sampling was obtained from the Ethics (Medical Research) Committee, Our Lady's Children's Hospital, Dublin.

Water samples

A total of 370 water samples were collected in three sampling sites located along the Dargle River (DRG1, DRG2 and DG3) and seven located in the tributaries: Swan (SWN), County Brook (CBR), Kilmacanogue (KMG1 and KMG2), Killough (KGH), Glencullen (GCU) and Glencree (GCR) rivers (Figure 2). Twenty-five water samples were obtained from the effluent of a Wastewater Treatment Plant in Enniskerry, Co. Wicklow (6,000 p.e.) after a secondary treatment with nutrient reduction, before discharging into the Dargle River. Samples were collected in sterile containers, transported on ice and stored at 4ºC until analysis. The levels of total coliforms, *E. coli* and Enterococci, were measured using Colilert-18 and Enterolert and Quanti-Tray/2000 (IDEXX Laboratories, UK), according to manufacturer's protocols.

DNA extraction and PCR amplification

DNA was extracted from 180-250 mg faeces using the QIAamp DNA Stool MiniKit (Qiagen, UK), according to the manufacturer's instructions. Water samples (100 ml) were filtered through a filter of 0.2 µm pore-size (Supor 200 PES, Pall Corporation, NY). Filters were placed in 0.5 ml GITC buffer (5 M guanidine thiocyanate, 100 mM EDTA [pH 8.0], 0.5% Sarkosyl) and frozen at -20ºC in lysis buffer until DNA extraction. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen), with some modifications, as reported previously (Gourmelon *et al.*, 2007).

Detection of the MST markers by the polymerase chain reaction (PCR) was performed. as described previously (Bernhard & Field, 2000; Dick *et al.*, 2005; Layton *et al.*, 2006; Lu *et al.*, 2008; Martellini *et al.*, 2005; Mieszkin *et al.*, 2009; Shanks *et al.*, 2008). PCR is a methodology in which template DNA is amplified in an exponential manner, thus allowing the detection of very small amounts of DNA in environmental samples. End-point PCR detects a DNA template in a qualitative manner (presence/absence), while quantitative PCR (qPCR) enumerates the number of template DNA molecules in a sample. The latter is quantified as gene copies (gc) per 100 ml.

Seven of these MST markers target host-specific Bacteroidales, one marker detects the 16S rRNA gene of *Catellicoccus marimammalium* and,

finally, two markers detect mitochondrial DNA from the host cells. Amplification with the universal primers F63 and 1389R (Marchesi *et al.*, 1998; Osborn *et al.*, 2000) was performed, in order to detect potential PCR inhibitors in the samples what may lead to false negative results. Controls included filtration blanks, DNA extraction blanks, no-template PCR control and PCR-positive control.

Plasmid standards

Plasmids, to be used as a positive control for PCR and as a quantification standard for qPCR, were constructed following amplification, using the HF183, CF128, HoF597, PF163, Pig-2-Bac, CowM3 and Gull markers. The amplicons were ligated into the pBluescript II KS (+) vector (Stratagene, CA, USA) using T4 Ligase and transformed into competent *E. coli* DH5α (Bethesda Research Lab). Recombinant bacteria were selected on Luria-Bertani medium containing ampicillin (100 µg/mL), on which 7 µl IPTG 20% (w/v) and 40 µl X-Gal (2% w/v) were plated 30 minutes prior to plating the transformants. Plasmids were extracted using High Pure Plasmid Isolation Kit (Roche). The nucleotide sequence of the inserts was determined to confirm that the correct target was cloned (GATC Biotech, Germany). The DNA concentration was quantified using NanoDrop ND-1000 (Thermo Scientific, DE, USA). Plasmid copy number was calculated based on the number of bp per plasmid. Plasmids were diluted in 10-fold serial dilutions to use as standards and maintained at -80ºC for a single use. PCR-positive controls for Ovmito and Pomito were obtained by extracting DNA from sheep and pig minced meat.

Quantification of land use

Geographic information systems (GISs) are used to integrate human activities into a river basin through land use mapping. The authors used ArcGIS 9.3 to visualise and analyse the CORINE land cover data from 2006 (CLC2006), in order to assess the different land uses in the Dargle catchment. Additional GIS data were obtained from Ordnance Survey Ireland (OSI). These layers included river segments, catchment and sub-catchment areas and digital elevation models.

Statistical analysis

The sensitivity and specificity for the eight microbial source tracking markers were calculated as previously defined (Gawler *et al.*, 2007). The positive predictive value or conditional probability that a particular source of faecal contamination was present when a water sample tested positive for the corresponding MST marker was calculated using Bayes' Theorem (Kildare *et al.*, 2007). Only positive samples in end-point PCR were analysed by qPCR. The detection limit was established through the analysis of serial dilution of faecal and water samples. The detection limits were 200 gc/ml for qHF183, qHoF597, CowM3 and AllBac, and in the case of the CF128 marker, the detection limit was higher at 1,000 gc/100 ml. Lower concentrations could be detected but not quantified accurately due to the variability when working with low concentration of DNA.

The quantitative values of the qPCR markers were $\log_{10}$ converted to achieve normality. Normal distribution was assessed using the Shapiro-Wilk Test, and Levane's Test was used to check homogeneity of variances. Converted data was used to perform correlation analysis through the software PASWStatistics Version 18.0.2 (IBM Corporation, Chicago, IL, US).

Results

Validation of MST markers for use in Ireland

MST markers to detect faecal pollution by humans, ruminants, sheep, horses, pigs and gulls were published before and during the course of this project (Bernhard & Field, 2000; Dick *et al.*, 2005; Lu *et al.*, 2008; Mieszkin *et al.*, 2009). In order to validate these for use in Ireland, 181 faecal samples from humans and a range of animals were collected, and subjected to DNA extraction and end-point PCR using oligonucleotides that specifically amplify a MST marker. To rule out that negative results were due to inhibition of PCR, all samples were amplified using general bacterial 16S rRNA primers. Samples of each animal source were collected from at least two different locations. In addition, DNA was extracted from raw and treated sewage.

In order to obtain a 95% confidence interval for the estimates of sensitivity and specificity, at least 20 faecal samples of target species were analysed. Sensitivity (r) and specificity (s) are defined as r=a/(a+c) and s=d/(b+d), where a is when a faecal DNA sample is positive for the PCR marker of its own species (true positive); b is when a faecal DNA sample is positive for a PCR marker of another species (false positive); c is when a faecal DNA sample is negative for a PCR marker of its own species (false negative); and d is when a faecal DNA sample is negative for a PCR marker of another species (true negative).

The performance of the MST markers was very good, achieving a sensitivity higher than 85% for most of the markers. In the case of the human marker HF183, a sensitivity of 73% was obtained when individual faecal samples were analysed, however, the sensitivity of the human marker was 100% using raw and treated sewage samples. The specificities of all markers were 87% or higher (Table 1).

Level MST markers in their hosts and in sewage

The levels of human (HF183), ruminant (CF128), cow (CowM3) and horse (HoF597) markers and total Bacteroidales (AllBac) were measured by qPCR in faecal samples from different sources (Figure 1, Table 2). The number of positive samples, as determined by qPCR, was higher than by end-point PCR, although most of these contained very low target DNA concentrations or were below the quantification limit.

The human HF183 marker displayed considerable variability within human samples ranging from 3.7 to 8.4 $\log_{10}$ gc/100 mg (Figure 1, Table 2), whereas more even and higher average levels were observed in samples from raw and treated sewage. The ruminant (CF128) MST levels were around 2.5 – 4.0 $\log_{10}$ higher in ruminant samples compared to faeces from humans and other animal sources (Figure 1, Table 2). In contrast to the human marker, the ruminant marker displayed low variability among individuals of the same group.

The levels of the cow marker (CowM3) were 2.3 – 3.2 $\log_{10}$ higher in faeces from cows and sheep than from other sources. Half of the sheep samples analysed were positive for this marker. The concentration of the horse (HoF597) marker was an order of magnitude (1.3 to 2.5 $\log_{10}$) higher in horse faeces than in faeces from other animal sources (Figure 1, Table 2). The levels of general Bacteroidales were high and similar for all the tested mammalian faecal samples

(Figure 1, Table 2). In contrast, the faecal samples from swans and gulls showed lower levels of the general Bacteroidales marker. The negative controls for sample processing, nucleic DNA extraction, and PCR and qPCR reactions were, in all cases, negative.

Table 1. Sensitivity and specificity of MST markers as determined by end-point PCR of DNA extracted from faecal samples and sewage. The number of positives samples (x) and total number (y) of samples are indicated as x/y.

	Human marker	Ruminant marker	Sheep marker	Horse marker	Pig marker			Gull marker
	HF183	CF128	Ovmito	HoF597	PF163	Pig-2-Bac	Pomito	Gull
Human	19/26	1/26	1/26	2/26	0/26	0/26	7/26	0/26
Cow[a]	2/22	22/22	0/22	1/22	0/22	0/22	0/22	0/22
Sheep[a]	5/25	25/25	25/25	1/25	4/25	0/25	0/25	0/25
Deer[a]	0/15	15/15	4/15	0/15	0/15	0/15	0/15	0/15
Goat[a]	0/2	2/2	0/2	0/2	½	0/2	0/2	0/2
Horse	3/27	5/27	0/27	26/27	6/27	0/27	0/27	0/27
Donkey	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Pig	6/20	8/20	0/20	2/20	20/20	15/20	20/20	0/20
Gull	1/26	0/26	0/26	0/26	1/26	0/26	0/26	22/26
Swan	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Peacock	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
% Sensitivity	**73**	**100**	**100**	**96**	**100**	**75**	**100**	**85**
% Specificity	**89**	**87**	**97**	**96**	**92**	**100**	**95**	**100**
Raw sewage	4/4	0/4	2/4	0/4	0/3	0/3	3/3	0/3
	100%	0%	50%	0%	0%	0%	100%	0%
Secondary treatment effluent	25/25	6/25	19/25	3/22	8/25	0/25	13/25	0/25
	100%	24%	76%	14%	32%	0%	52%	0%

[a]Ruminant species

To statistically verify whether differences existed when comparing the qualitative end-point PCR and the levels of host-marker levels among the corresponding target and non-target samples, as determined by qPCR, a Chi-squared and independent sample Kruskal-Wallis Test ($P < 0.05$) analysis was used. The differences within the target and non-targeted samples were statistically significant for all the markers evaluated in the study. Thus, the MST markers tested are potentially useful to discern among different faecal samples.

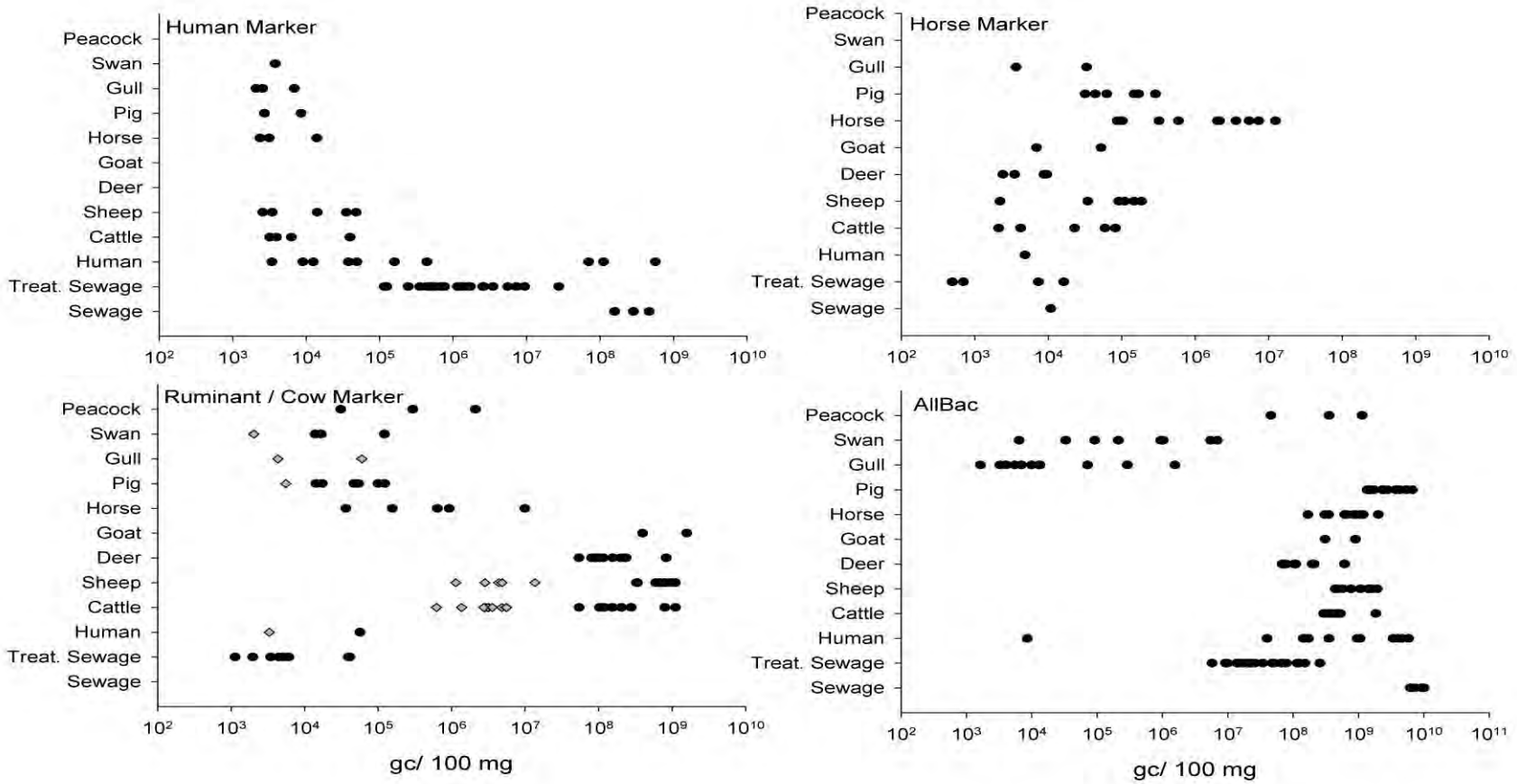

Figure 1. Quantification of MST markers in individual faecal samples from humans and a range of animals. The markers used were human marker (HF183), horse marker (HoF597), ruminant marker (CF138), cow marker (CowM3) and general Bacteroidales (AllBac). gc: gene copy.

Table 2. Quantification of MST markers in individual faecal samples from humans and a range of animals. The markers used were human marker (HF183), horse marker (HoF597), ruminant marker (CF138), cow marker (CowM3) and general Bacteroidales (AllBac). n.a.: non-applicable.

	Human marker qHF183				Ruminant marker qCF128				Cow marker CowM3			
	No. positive samples / Total samples	% of detection	Median Log_{10}	95% C.I. Log_{10}	No. positive samples/ Total samples	% of detection	Median Log_{10}	95% C.I.	No. positive samples / Total samples	% of detection	Median Log_{10}	95% C.I Log_{10}
Human	**10/12**	**83%**	**4.95**	**3.72-8.43**	1/12	8%	4.76	n.a.	1/10	10%	3.52	n.a.
Cow[a]	4/10	40%	3.70	3.52-4.48	**10/10**	**100%**	**8.25**	**7.86-8.98**	**10/10**	**100%**	**6.50**	**5. 95-6.75**
Sheep[a]	5/10	50%	4.15	3.44-4.66	**10/10**	**100%**	**8.83**	**8.52-9.02**	5/10	50%	6.64	6.14-7.04
Deer[a]	0/9	0%	n.a.	n.a.	**10/10**	**100%**	**8.04**	**7.81-8.68**	0/10	0%	n.a.	n.a.
Goat[a]	0/2	0%	n.a.	n.a.	**2/2**	**100%**	**8.90**	**n.a.**	0/2	0%	n.a	n.a.
Horse	3/11	27%	3.5	3.39-4.08	5/10	50%	5.81	4.69-6.79	0/10	0%	n.a.	n.a.
Donkey	0/1	0%	n.a.	n.a.	1/1	100%	4.37	n.a	0/1	0%	n.a.	n.a.
Pig	2/11	18%	3.68	3.46-3.91	6/11	55%	4.70	4.18-5.07	1/10	10%	3.75	n.a.
Gull	3/11	27%	3.41	3.33-3.80	0/11	0%	n.a.	n.a.	2/10	20%	4.21	3.69-4.72
Swan	1/8	12%	3.58	n.a.	3/8	38%	4.23	4.15-5.00	1/8	12%	3.31	n.a.
Peacock	0/3	0%	n.a.	n.a.	3/3	100%	5.47	4.59-6.24	0/3	0%	n.a.	n.a.
Raw sewage	**4/4**	**100%**	**8.33**	**8.20-8.64**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a	n.a.	n.a.
Secondary treatment effluent	**25/25**	**100%**	**5.89**	**5.07-7.34**	8/25	32%	3.71	3.15-4.61	n.a	n.a.	n.a.	n.a.

	Horse marker qHoF597				Bacteroidales marker AllBac			
	No. positive samples /Total samples	**% of detection**	**Median Log_{10}**	**95% C.I Log_{10}**	**No. positive samples/ Total samples**	**% of detection**	**Median Log_{10}**	**95% C.I Log_{10}**
Human	1/11	9%	3.68	n.a.	**12/12**	**100%**	**8.76**	**5.95-9.71**
Cow[a]	5/10	50%	4.36	3.39-4.89	**10/10**	**100%**	**8.62**	**8.48-9.02**
Sheep[a]	6/10	60%	5.00	3.64-5.24	**10/10**	**100%**	**9.09**	**8.66-9.29**
Deer[a]	4/10	40%	3.74	3.41-3.97	**10/10**	**100%**	**8.03**	**7.85-8.58**
Goat[a]	2/2	100%	4.28	n.a.	**2/2**	**100%**	**8.72**	**n.a.**
Horse	**10/10**	**100%**	**6.32**	**4.94-6.99**	**11/11**	**100%**	**8.92**	**8.36-9.19**
Donkey	1/1	100%	4.83	n.a.	**1/1**	**100%**	**7.95**	**n.a.**
Pig	6/11	55%	4.98	4.53-5.40	**10/10**	**100%**	**9.42**	**9.14-9.78**
Gull	2/11	18%	4.04	n.a.	**11/11**	**100%**	**4.00**	**3.36-5.83**
Swan	0/8	0%	n.a.	n.a.	**8/8**	**100%**	**5.65**	**4.06-6.80**
Peacock	0/3	0%	n.a.	n.a.	**3/3**	**100%**	**8.55**	**7.75-9.01**
Raw sewage	n.a.	n.a.	n.a.	n.a.	**4/4**	**100%**	**9.92**	**9.80-10**
Secondary treatment effluent	4/20	20%	3.67	3.22-4.63	**23/23**	**100%**	**7.35**	**6.97-8.17**

Field testing of validated MST markers

The validated MST markers were tested in the Dargle catchment, which is located just south of the Dublin-Dun Laoghaire urban area, at the north-eastern end of the Wicklow Mountains.

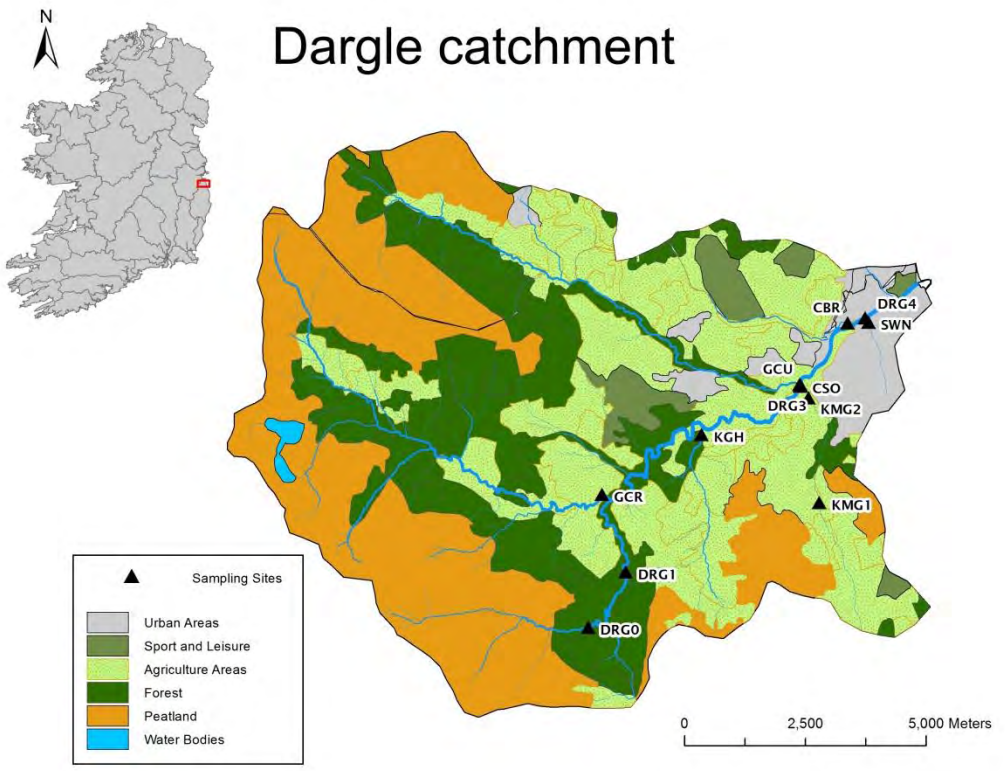

Figure 2: Localisation of the sampling stations and land use in the Dargle catchment. DRG, Dargle; GCR, Glencree; KGH, Killough; KMG, Kilmacanogue; GCU, Glencullen; CBR, County Brook; SWN, Swan.

Table 3: Land use in the Dargle catchment. Station legend as in Figure 2.

	DRG0/1	DRG2/3	KGH	KMG1	KMG2	CBR	SWN/DRG4	GCU	GCR
Urban	0%	0%	0%	0%	1%	2,4%	96,2%	4%	0%
Sport	0%	10,1%	0%	3,1%	2,1%	26,1%	0%	2,3%	0%
Agriculture	3%	52,5%	70,6%	55,1%	59,1%	60,1%	2,2%	36,8%	14,4%
Forest	31,6%	33,1%	5,3%	1,7%	6,8%	11,4%	1,3%	26,4%	35,5%
Bogs	65,4%	4,3%	24,1%	40,1%	31%	0%	0,3%	30,5%	48,8%
Lakes	0%	0%	0%	0%	0%	0%	0%	0%	1,3%

It covers 121 km^2 and discharges to the sea through Bray harbour. There are extreme variations in climate within the catchment. In the eastern, coastal part of the catchment, annual rainfall is ca. 800 mm, there are approximately 185 days with rain per year, and monthly mean rainfall figures range between 50 mm in summer and 70 mm in winter. Mean daily temperatures lie between 5ºC in winter and 15ºC in summer; mean daily sunshine duration lies between 1.6 hrs per day in winter and 5.6 hrs in summer. In the mountains, rainfall is 1,500–2,000 mm per year, with snow-cover lasting a month; daily temperatures are much lower and sunshine hours fewer. The village of Enniskerry in the centre of the catchment receives rainfall levels of 900–1,300 mm per year based on 90–140 rain days per year, when between 1 and 60 mm precipitation is delivered. For the purpose of this study, the catchment has an attractive combination of the following features:

(a) land area and number of sub-catchments;
(b) wide range of land use categories;
(c) readily accessible and representative water sampling and gauging sites;
(d) sampling sites within a workable distance of the laboratory;
(e) meteorological data readily available.

The Dargle catchment was sampled at 13 stations during the lifetime of the project (Figure 2). In addition to MST markers, the levels of faecal indicator bacteria (FIB), i.e. *E. coli* and Enterococci, were determined to determine the level of faecal pollution in the sub-catchments of the Dargle. The highest levels of FIB (Figures 3 and 4) were obtained from sampling stations surrounded by urban areas: The Swan (SWN) and Kilmacanogue (KMG2) Rivers, and the Dargle River after the confluence of the outfall (CSO) of the Enniskerry WWTP (DRG3). Interestingly, there was a 10-fold increase in FIB levels in the Kilmacanogue River when comparing the sampling sites KMG1 and KMG2, which are separated by approximately 2 km. Lower levels were detected in County Brook (CBR), Killough River (KGH), Kilmacanogue River upstream of the town (KMG1), and the Dargle before the Enniskerry WWTP outfall (DRG2). The most pristine waters are found in the Glencullen (GCU) and Glencree (GCR) Rivers, and the upstream reaches of the Dargle River (DRG0 and DRG1).

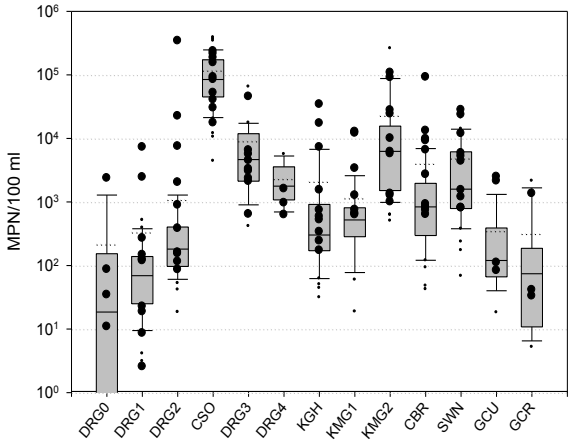

Figure 3. Box plots showing E. *coli* levels (log MPN/100ml) for the sampling sites in the Dargle catchment. The median, 25% and 75% percentiles are shown in the grey boxes. The lines show the means. Black dots show the levels obtained after heavy rains (Sampling + previous day >7.5 mm).
Station legend as in Figure 2.

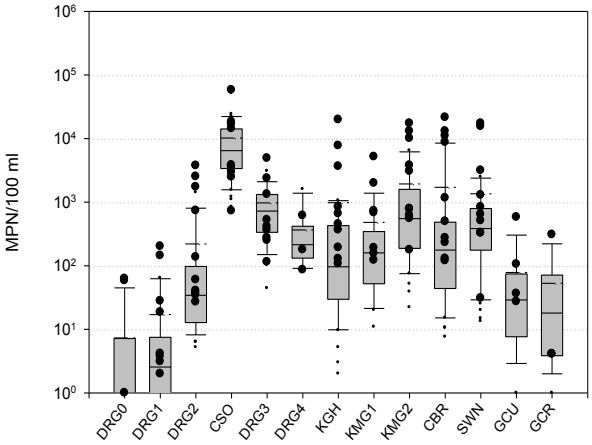

Figure 4. Box plots showing Enterococci levels (log MPN/100ml) for the sampling sites in the Dargle catchment. The median, 25% and 75% percentiles are shown in the grey boxes. The lines show the means. Black dots show the levels obtained after heavy rains (Sampling + previous day >7.5 mm).

Microbial source tracking with end-point PCR

The Dargle catchment is thus very diverse in the levels of faecal contamination (Figures 3 and 4), which is to be expected based on its land use (Figure 2, Table 3). In order to analyse the sources of pollution and field test the MST markers, water samples were taken over a two-year period and analysed using end-point PCR, which yields positive/negative results.

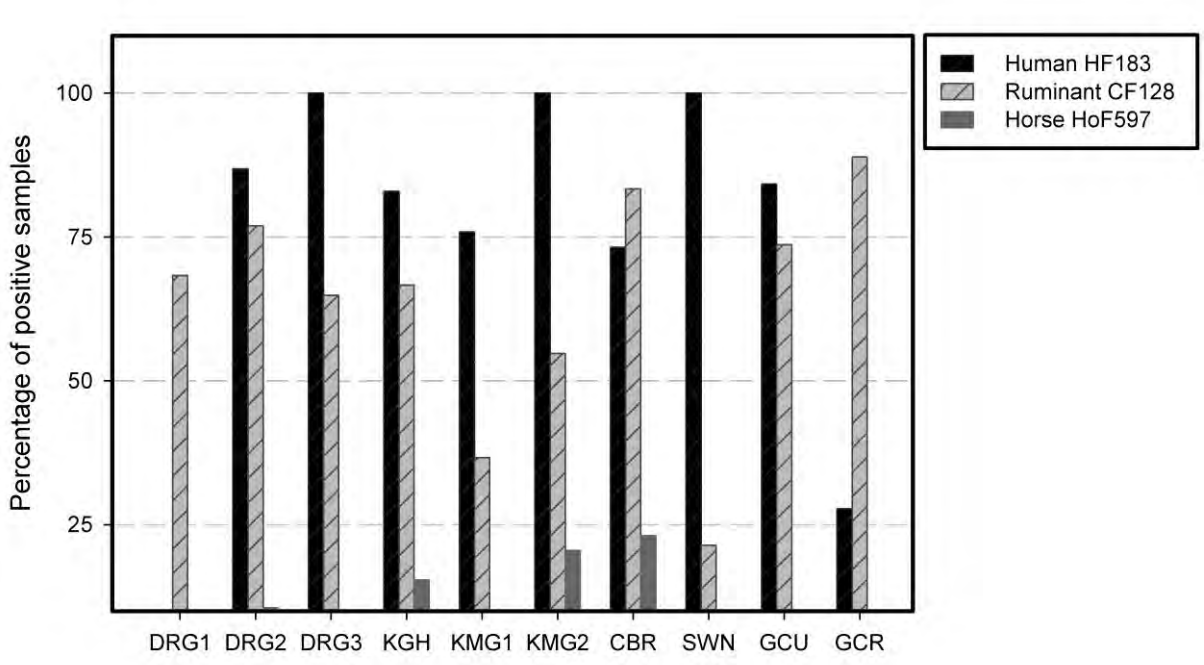

Figure 5: Microbial source tracking analysis of the Dargle catchment using end-point PCR. Shown are the percentages of positive samples for the human (HF183), ruminant (CF128) and horse (HoF597) MST markers.

The presence/absence of the human (HF183), ruminant (CF128) and horse (HoF597) MST markers were evaluated by end-point PCR in the Dargle catchment, since these target the main pressures in the areas of interest (human, ruminant and equine). The sampling stations DRG0 and DRG4 were not included. The human markers were detected in all samples collected at the DRG3, KMG2 and SWN sampling stations (Figure 5). The DRG3 site is impacted on by the effluent from the Enniskerry WWTP, which is located 30 metres upstream of the sampling site. The KMG2 site is located in Kilmacanogue, downstream of Kilmacanogue town. The Swan River traverses several housing estates in Bray. The human marker was also detected with high frequency (more than 80% of positive samples) in DRG2, KGH and GCU, but was virtually absent in samples taken at the DRG1 and GCR stations, which are located in the pristine areas of the Dargle catchment (Figure 2, Table 3). The ruminant marker was less prevalent than the human marker in water samples. CF183 was detected in more than 60% of the sampling sites surrounded by agricultural areas: DRG1, DRG2, DRG3, KGH, CBR, GCU and GCR, but was virtually absent in the Swan River (SWN), which flows through an almost exclusive urban area (Figure 2, Table 3). The horse marker (HoF597) showed a very low prevalence along the catchment. However, its presence stood out at the KMG2 and CBR sampling sites (more than 20%); several horse riding stables are located upstream from these sampling sites.

Because all PCR assays showed some levels of cross-reaction in faecal samples, using Bayesian statistic, the probability of detecting faeces from the target-host within given water samples was estimated. Given the prevalence of the three markers in the catchment, and the sensitivity and specificity calculated through the analysis of faecal samples (Table 1), the conditional probability of having human pollution when the

HF183 marker is detected was 0.95. The conditional probabilities for the ruminant marker (CF128) and horse marker (HoF597) were 0.93 and 0.75 respectively.

Seasonality of MST marker distribution

In order to determine whether the time of year had an impact on the number of samples that tested positive for a particular MST marker, the samples were grouped according to season (Figure 6). The presence of the human marker for faecal pollution did not appear to be influenced by the time of year in which the sample was taken. This is not unexpected, since human pollution is more than likely the point-source of pollution from sewerage misconnections, faulty septic tanks and the outfall of the Enniskerry WWTP. In contrast, a number of agriculturally-impacted sites displayed seasonal effects. For example, the majority of samples testing positive for the ruminant marker in the upstream reaches of the Dargle (DRG1), and in the Kilmacanogue (KMG1, 2) and Killough (KGH) Rivers, were taken in summer and autumn, with only a small percentage testing positive in winter. This most likely reflects farming practices, including the gathering of animals in stables in winter and the banning of spreading of slurry from October until January.

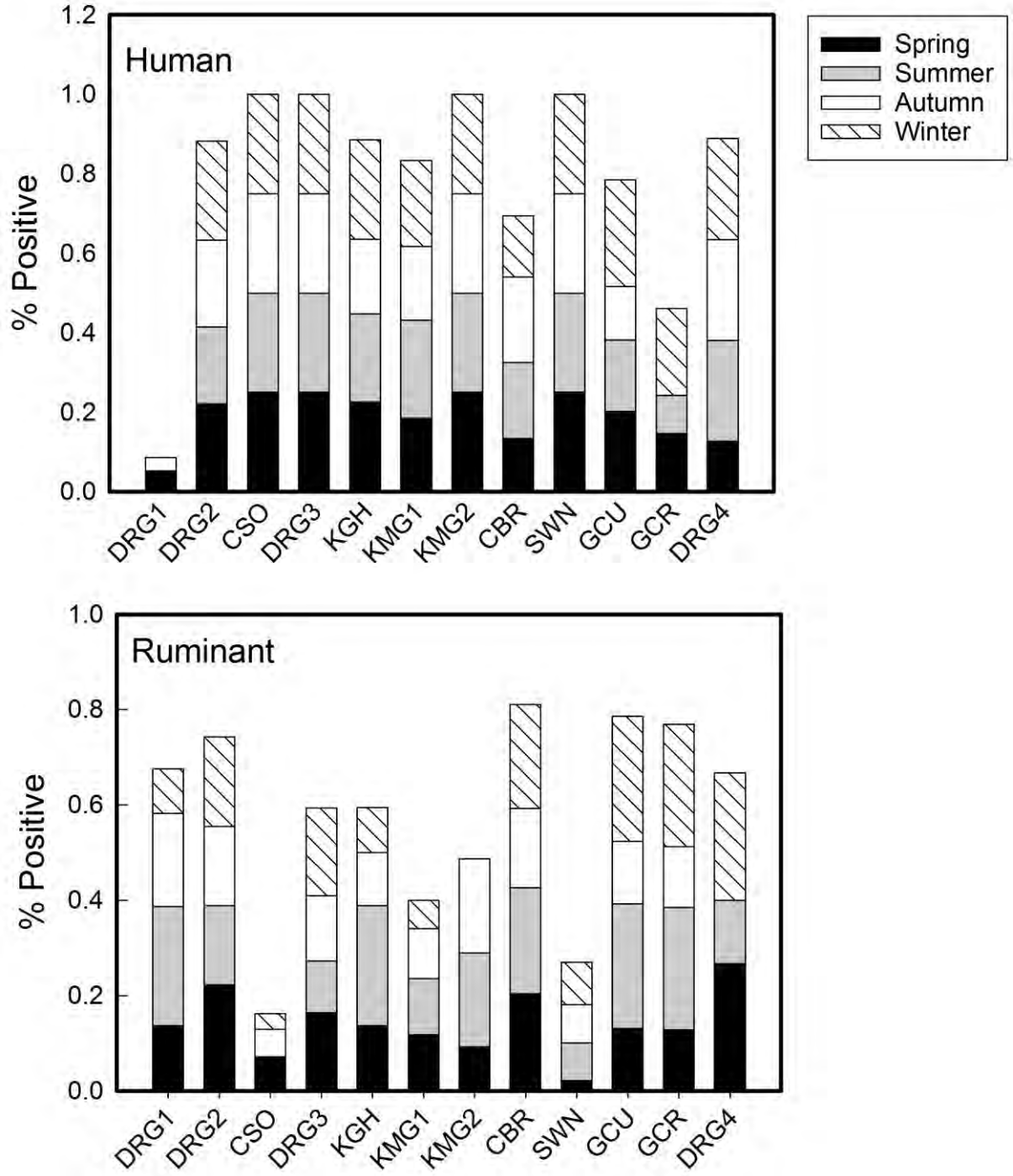

Figure 6. Percentage of positive samples for the human marker (HF183) and the ruminant marker (CF128) in the different sampling sites along the Dargle Catchment and among the different seasons.

Microbial source tracking using qPCR

It has been suggested that the level of Bacteroidales species in water samples, as determined by qPCR, may serve as a substitute for FIB. To evaluate this, the authors analysed water samples taken at all sampling stations using the AllBac marker, which is used to amplify Bacteroidales 16S rRNA genes. The level of this general Bacteroidales marker was higher than 10^4 gc/100 ml for all the measurements (Figure 7), and their number correlated well with the

levels of *E. coli* (r: 0.843; P < 0.001; n: 203) and Enterococci (r: 0.791; P < 0.001; n: 198). However, although the levels of the Bacteroidales marker were correlated to those of *E. coli* and Enterococci, their levels were also high at upstream sampling sites in the Dargle and Glencree Rivers (DRG1, GCR), which displayed none or very low concentrations of *E. coli* and Enterococci. This strongly suggests that the marker detects non-intestinal environmental, as well as intestinal Bacteroidales species. The authors therefore conclude that the enumeration of Bacteroidales species is not suitable as an indicator of faecal pollution. These findings are in agreement with recent studies (van der Wielen & Medema, 2010; Vierheilig *et al.*, 2012).

The levels of the human MST marker were high (10^5-10^6 gc/100 ml) in sampling sites with anthropogenic impact: the Swan (SWN) and Kilmacanogue (KMG2) Rivers, and the Dargle after the Enniskerry WWTP outfall (CSO, DRG3) (Figure 7). Intermediate levels of the human marker, between 10^3-10^4 gc/100 ml, were detected in sampling sites with medium anthropogenic impact (Killough [KGH], County Brook [CBR], Glencullen [GCU], Kilmacanogue [KMG1] and Dargle [DRG2]). The levels of the human marker were very low at the Waterfall Bridge in the Dargle (DRG1) and in the Glencree (GCR) River.

The levels of human marker (qHF183) showed very similar trends as those observed for *E. coli* and Enterococci (Figures 3, 4, 7 and Table 4). High levels of the human marker HF183 were detected in KMG2, SWN and DRG3, which were also 100% positive for the human marker, as determined with end-point PCR (Figure 5, Table 4). These sampling stations also had the highest levels of *E. coli* and Enterococci (Figures 3 and 4, Table 4). The levels of the human marker correlated very well with the levels of *E. coli* and AllBac in samples taken from KMG2 (r = 0.474, P = 0.019, n = 24; r = 0.858, P <0.001, n = 24) and DRG3 (r = 0.423, P = 0.044, n = 23; r = 0.735, P <0.001, n = 23), and with Enterococci in KMG2 (r = 0.541, P = 0.006, n = 24). The levels of the human marker (HF183) were 1.3 to 2 $\log_{10}$ lower in DRG2, KGH, KMG1, CBR and GCU, and were below the detection limit or appeared at very low levels in GCR and DRG1.

There was a fairly even distribution of the levels of the ruminant marker along the different sampling points of the Dargle catchment (~ 10^4 molecules/100 ml) (Figure 7). However, in sampling points with an agricultural impact (KGH, KMG1, KMG2 and CBR, Figure 2 and Table 3), the levels of the ruminant marker soared sporadically, often related to rainfall or unknown events, such as accidental spills.

Table 4. Median and 95% confidence interval (C.I.) of the log_{10} transformed data from the MST markers of Dargle samples expressed by the evaluated qPCR. Positive samples / Total samples (PS/TS), percentage of positive samples evaluated by PCR.

	DRG1		DRG2		DRG3		KGH		KMG1		KMG2	
	Median	**95% C.I.**	**Median**	**95% C.I.**	**Median**	**95% C.I.**	**Median**	**95% C.I.**	**Median**	**95% C.I.**	**Median**	**95% C.I.**
E. coli	1.84	0.64-2.71	2.27	1.72-3.36	3.65	2.84-4.36	2.48	1.71-3.85	2.70	1.84-3.47	3.80	2.81-5.03
Ent	0.57	<1-1.94	1.59	0.80-3.16	2.83	2.05-3.40	1.94	0.73-3.55	2.22	1.18-3.29	2.81	1.72-4.00
qHF183	2.68	1.61-3.67	3.48	2.48-4.15	4.89	4.07-5.79	3.70	2.56-4.71	3.47	2.41-4.57	5.35	4.44-6.35
qCF128	3.65	3.16-4.52	3.84	2.96-5.01	4.11	3.16-4.89	4.18	3.20-5.36	4.02	2.84-5.33	4.87	3.93-5.88
AllBac	5.05	4.53-5.80	5.62	5.13-6.55	6.35	5.70-6.97	5.55	4.90-6.58	5.88	5.50-6.34	6.59	6.07-7.66
	PS/TS	**% of positive**	**PS/TS**	**% of positive**	**PS/TS**	**% of positive**	**PS/TS**	**% of positive**	**PS/TS**	**% of positive**	**PS/TS**	**% of positive**
HF	3/40	7.5%	33/38	86.8%	36/36	100%	34/41	82.9%	22/29	75.9%	41/41	100%
CF	28/41	68.3%	30/39	76.9%	24/37	64.9%	28/42	66.7%	11/30	36.7%	23/42	54.8%
HoF	2/39	5.1%	4/38	10.5%	3/37	8.1%	6/39	15.4%	2/30	6.7%	8/39	20.5%

	CBR		SWN		GCU		GCR	
	Median	**95% C.I.**	**Median**	**95% C.I.**	**Median**	**95% C.I.**	**Median**	**95% C.I.**
E. coli	2.98	1.97-3.99	3.20	2.26-4.31	2.22	1.67-3.02	2.06	0.85-3.17
Ent	2.34	1.04-4.04	2.59	1.13-4.14	1.56	0.54-2.44	1.49	0.41-2.23
qHF183	3.81	2.64-4.62	5.29	4.42-7.32	3.63	2.96-4.66	3.20	2.38-3.67
qCF128	4.41	3.37-6.44	3.94	2.42-4.92	4.01	3.13-4.79	4.18	3.48-4.98
AllBac	5.89	5.38-7.13	6.60	5.88-7.66	5.49	5.06-6.14	5.38	5.03-5.93
	PS/TS	**% of positive**	**PS/TS**	**% of positive**	**PS/TS**	**% of positive**	**PS/TS**	**% of positive**
HF	30/41	73.2%	41/41	100%	16/19	84.2%	5/18	27.8%
CF	35/42	83.3%	9/42	21.4%	14/19	73.7%	16/18	88.9%
HoF	9/39	23.1%	3/39	10.3%	1/18	0%	0/18	0%

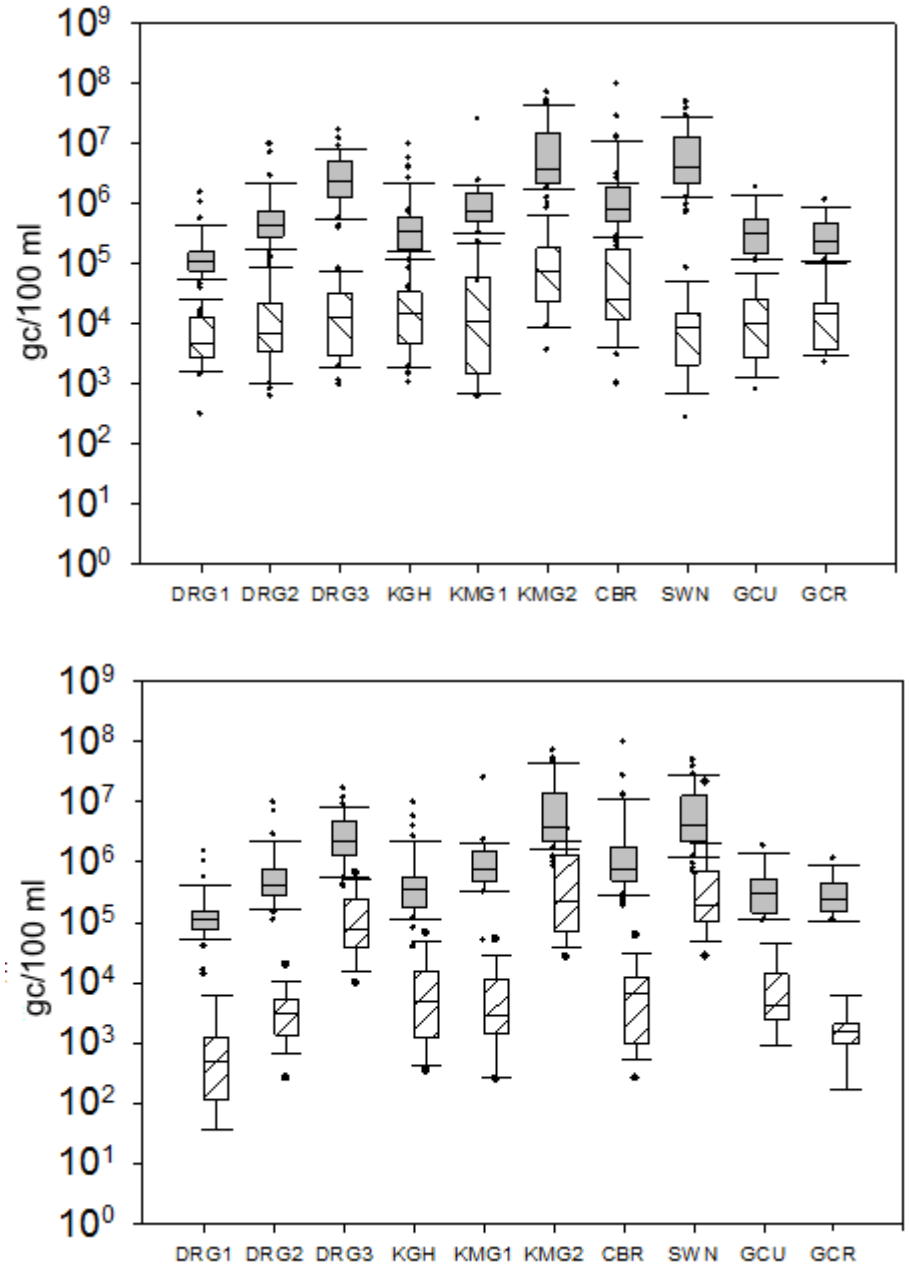

Figure 7: Box plots showing the MST marker levels of the general Bacteroidales (grey), human (white-striped) and ruminant (white-striped) markers in the Dargle catchment over a two-year period, as determined by qPCR. The top panel compares the general Bacteroidales marker to that of the human marker; the bottom panel shows the comparison between the general Bacteroidales marker to the ruminant marker. The legend for the sampling stations is as in Figure 2.

Forensic use of microbial source tracking

Swan River

The Swan River was chosen to demonstrate the usefulness of microbial source tracking as a forensic tool to localise the biological and geographical origins of faecal contamination, as well as to provide an estimate of the amount of faecal-derived matter present.

The Swan River has its source in the Kilruddery Estate and discharges into the Dargle after passing through a number of housing estates (Figure 8). The Swan catchment has approximately 3,800 households. The average household has 2.8 persons (as estimated by the EPA), i.e. there are approximately 10,640 people living in the catchment.

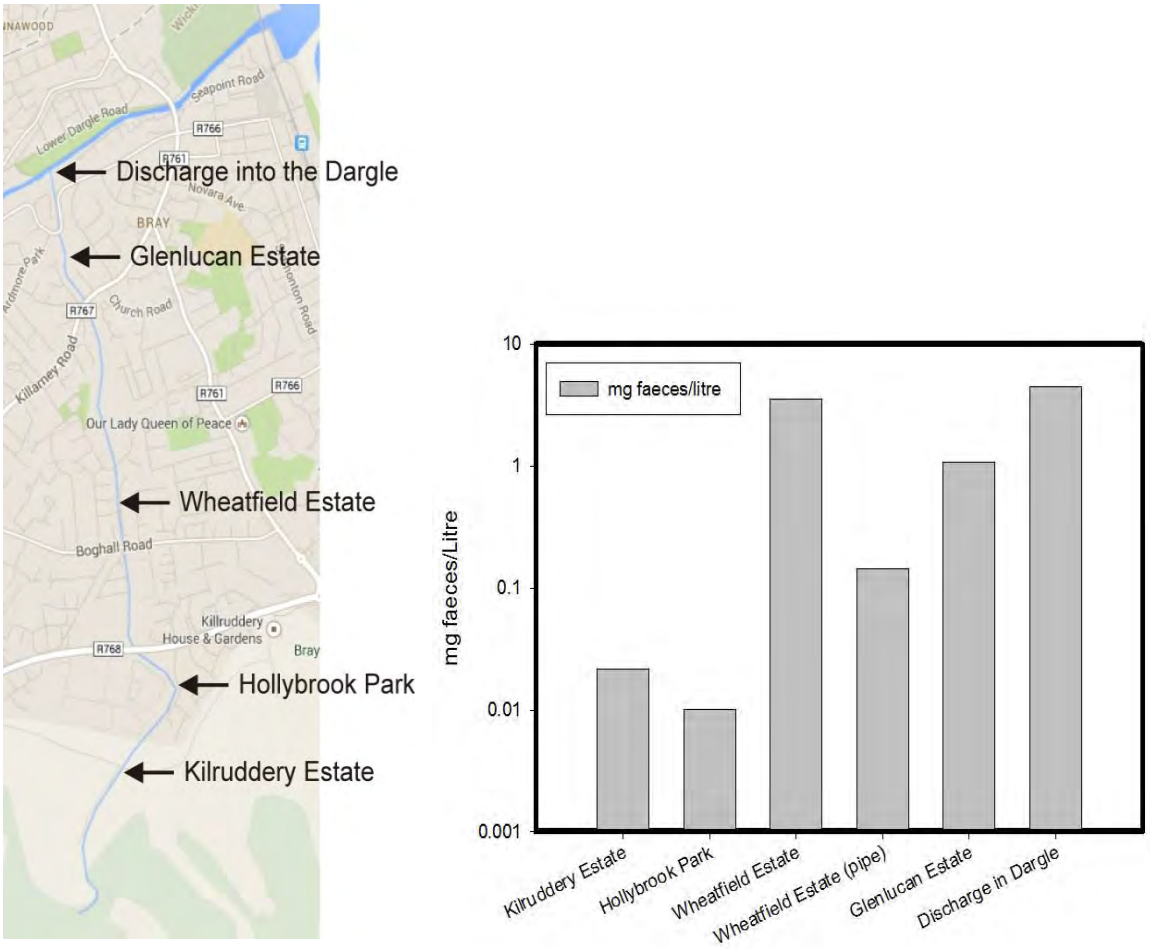

Figure 8: Human faecal-derived matter in the Swan River. The arrows in the map indicate sampling stations. Samples were taken at two locations in the Wheatfield Estate: in the Swan River and in a pipe discharging into the Swan River. The latter is indicated in the bar chart by 'pipe'.

The majority of households are connected to the public sewerage system, however, the catchment also has a number of (probably old) septic tanks. The average water flow of the Swan River is 1.06×10^7 litres per day (as determined by one of the gauges installed in the Dargle catchment for the EU Interreg-funded Smartcoasts project, www.smartcoasts.eu).

During this project, water samples from the Swan River were collected where the river discharges into the Dargle. This project identified this river as one of the most polluted in the Dargle catchment (Figures 3 and 4). MST, using end-point PCR, had shown that this river was almost exclusively impacted on by human faecal pollution (Figure 5). Quantitative PCR analysis showed that the level of the human marker (HF183) was one of highest in the Dargle catchment (Figure 7, Table 4). The authors therefore decided to analyse the Swan River at various stations between its origin in the Kilruddery Estate and its discharge into the Dargle. DNA was extracted from these water samples and subjected to qPCR, to determine the level of the human MST marker (HF183). Since the authors also determined the level of this marker per gramme of human faeces (gc/gram faeces; Figure 1, Table 2), they used this information to make an estimation of the amount of faecal matter that is required to account for the levels of the human MST marker in the Swan River.

The amount of faecal-derived matter is very low close to the source of the Swan River in the Kilruddery Estate, and remains low in Hollybrook Park, but increases by more than two orders of magnitude when the Swan River passes through the Wheatfield Estate to around 4 mg/litre (Figure 8). At the point of discharge into the Dargle, the amount of human faecal-derived matter is 4.5 mg/litre (Figure 8), with an average flow of $1.06x10^7$ litre/day. This means that the Swan discharges 48 kg of faecal-derived matter per day into the Dargle. Assuming that a person produces on average 150 grammes of faeces a day, this means that faecal-derived matter equivalent to 113 out of 3,800 households, ends up in the Swan River, which accounts for 3% of the total faecal production in the Swan catchment.

Kilmacanogue River

A similar situation as in the Swan River occurs in the Kilmacanogue River, in between the sampling stations KMG1 and KMG2 (Figure 2). While this river is relatively clean at KMG1, the level of FIB increased by one to two orders of magnitude at the KMG2 sampling station (Figures 3 and 4), resulting in comparable pollution levels as in the Swan River. This is accompanied by an increase of at least two orders of magnitude in the level of the human MST marker (Figure 7, Table 4). These data demonstrate the presence of a major source of human pollution in the Kilmacanogue River, in between stations KMG1 and KMG2.

Persistence of microbial source tracking markers

Bacteroidales species are obligate anaerobic bacteria of the mammalian intestinal track. Following release into the catchment, they will be subject to suboptimal growth temperatures, an aerobic environment, and grazing by protozoa, resulting in their progressive disappearance. However, as was noted in the above, the MST markers target different Bacteroidales species, and these may therefore be differentially resistant to these adverse conditions. To gain insight into the differential die-off rates of these MST markers, mesocosm experiments were carried out with non-sterilised Dargle river water. To this end, containers containing Dargle river water (200 litres each) were spiked with a mixture of human, equine and bovine faeces. The mesocosms were incubated on the roof of the UCD Science building (average temperature 8.8ºC), such that they experienced similar lighting and temperature conditions as the Dargle. The containers were continuously mixed and aerated. The levels of *E. coli* and

Enterococci, as well as those of the human, horse and ruminant MST markers were determined over a 250-hour period (Figure. 9).

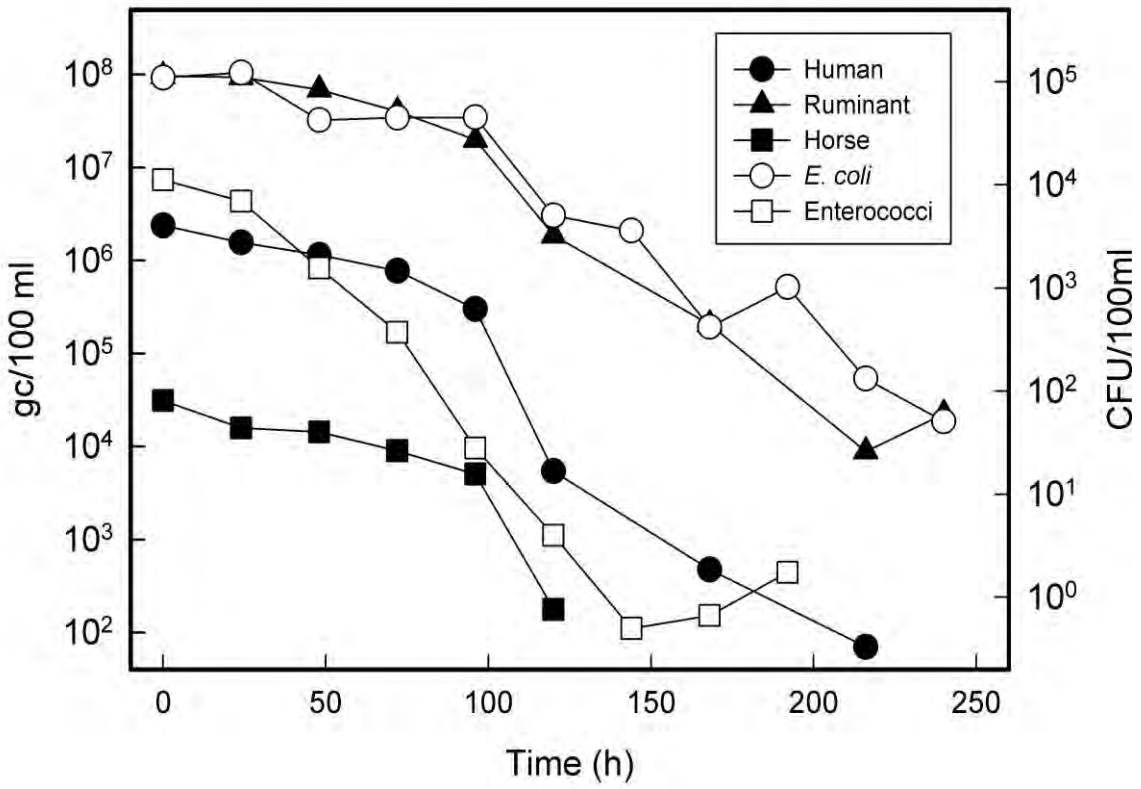

Figure 9: Persistence of the human, ruminant and equine MST markers (gc/100 ml) and of E. *coli* and Enterococci (CFU/100ml) in a 200 L mesocosm, following inoculation with a mixture of human, bovine and equine faeces. The mesocosm contained non-sterilised Dargle river water and was aerated throughout the experiment.

The faecal indicator bacteria Enterococci and *E. coli* disappeared first, with T_{90} values of 32 and 93 hours respectively (Figure 9, Table 5). The die-off rates for the MST markers were similar to each other, but persisted significantly longer than those of the FIB (Figure 9, Table 5).

Table 5: Persistence of the human (HF183), ruminant (CF128) and horse (HoF597) MST markers, and E. *coli* and Enterococci in a 200-litre mesocosm, as determined by qPCR. Shown are the T_{90} values in hours and days.

	T_{90} (h)	T_{90}(days)
Human	113	4.8
Ruminant	138	5.7
Horse	131	5.5
E. coli	93	3.9
Enterococcus	32	1.3

A major difference between the die-off rates of the FIB and MST markers is that the former are based on the detection of live cells, whereas the latter are based on detection of DNA by PCR. The qPCR method thus detects both live and dead cells, which may account for the higher T_{90} values.

To investigate this, samples were treated with propidium monoazide (PMA) prior to DNA extraction and PCR. PMA forms a covalent bond with double-stranded DNA, which prevents amplification by PCR. PMA cannot penetrate the membrane of live cells, but readily enters dead cells. PMA treatment therefore prevents the amplification of free DNA and DNA of dead but not of live cells, resulting in the detection of live cells only.

Table 6: Persistence of the human (HF183), ruminant (CF128) and horse (HoF597) markers in a 200-litre mesocosm inoculated with human, ruminant and equine faeces, as determined by end-point PCR. Live: Samples treated with propidium monoazide; only live cells are detected. All: Samples not treated with propidium monoazide; both live and dead cells are detected. 1: Marker is present, 2: marker is present in only one of the duplicate samples, 0: marker is absent.

		Time following inoculation (h)										
		0	24	48	72	96	120	144	168	192	216	240
Human	All	1	1	1	1	1	0	0	0	0	0	0
	Live	1	1	2	0	0	0	0	0	0	0	0
Ruminant	All	1	1	1	1	1	0	0	0	0	0	0
	Live	1	1	0	0	0	0	0	0	0	0	0
Horse	All	1	1	1	1	1	0	0	0	0	0	0
	Live	1	0	2	0	0	0	0	0	0	0	0

This approach was applied to end-point PCR analysis, which showed that MST markers were detectable up to 96 hours following inoculation without PMA treatment. However, the human and ruminant MST markers (Table 6) could only be detected up to 24 hours following inoculation of the mesocosms with faeces, when the samples were treated with PMA before DNA extraction. The horse marker disappeared between inoculation and 24 hours after inoculation. This experiment shows that the die-off rate for living cells is higher than the T_{90} determined by qPCR, which reflects both living and dead cells (Table 6).

The effect of weather events on water quality

(*This work package was added as a six-months', no-cost project extension, following completion of the project.)*

During this project, the Dargle catchment was not instrumented with flow and rain gauges, nor was a hydrodynamic catchment model available. In collaboration with Dr. John O'Sullivan (UCD), an integrated catchment near-shore hydrodynamic model of the Dargle catchment was developed, describing the impact of, amongst others, weather events on the levels of FIB in the catchment and ultimately at the compliance point at Bray South Promenade. Towards the end of this project, the Dargle catchment was instrumented with flow gauges at 10 locations, as well as with rain gauges and weather stations, which provided the data for a hydrological catchment model. The hydrological data were complemented by an intensive field work programme, which determined the levels of faecal indicator bacteria (FIB), Enterococci and *E. coli*, during normal flow conditions and during storm (high flow) events (Figure 10).

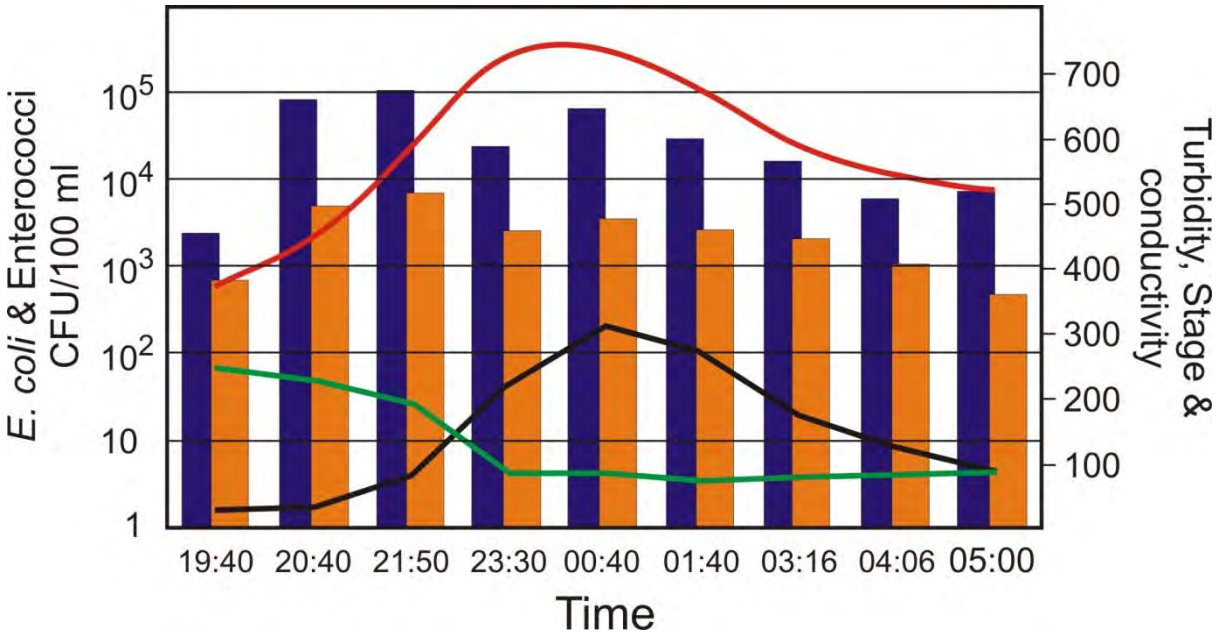

Figure 10: Example of hydrological and microbiological data obtained during heavy rainfall in 2012 at the Glencullen sampling station in the Dargle catchment. Blue bar, Escherichia coli; orange bar, Enterococci; red line, stage (mm); green line, conductivity (µS/cm); black line, mean turbidity (NTU). Time of day is indicated (hr).

These data allowed modelling of faecal contamination of the catchment and the effects weather events have on the input of contamination into the catchment. In addition to these samples, the authors also collected water samples for DNA extraction to determine the levels of MST markers. The data clearly show that bad weather events resulting in an increase in waterflow, results in a rapid increase in the number of *E. coli* and Enterococci by about two orders of magnitude, and is accompanied by an increase in turbidity. However, while the flow rate and turbidity rapidly return to their original levels, the number of colony forming units (CFUs) of *E. coli* and Enterococci remain high (Figure 10).

Conclusions and Recommendations

A major problem facing water quality managers is that, although they can identify that a water body is contaminated, they frequently do not know what the cause of this pollution is. This information is a critical component in the decision-making process aiming to identify the appropriate corrective measures. For example, a water body polluted by cattle cannot be remediated by investing in wastewater treatment systems. Furthermore, the EU Bathing Water Directive requires identification and assessment of causes of pollution that might affect bathing waters and impair bathers' health.

This study focused on the use of Microbial Source Tracking (MST) as a tool to identify the biological and geographical origins of faecal pollution in Ireland. The study shows that the MST markers identifying human, equine, ruminant, porcine, ovine and gull faecal pollution have high sensitivity and specificity, when applied to a panel of Irish faecal samples from humans, sewage and a range of animals using a qualitative approach (end-point PCR). These data were confirmed when quantitative PCR was employed on the same samples, which determines the level (gene copies/gramme of faeces) of the MST markers, showing that these were several orders of magnitude higher in target species than others. This quantitative approach also showed that the levels of these markers in individuals may significantly differ, particularly in humans. However, human pollution is rarely due to a single individual. The levels of this marker in raw and treated sewage showed a more even and consistent level, demonstrating that variability amongst individuals will not hamper the use of this marker in identifying human faecal pollution. Mesocosmos studies showed that once released in water, the MST markers do not persist but disappear, with T_{90} values ranging from 4.8 to 5.7 days. The MST markers therefore are indicative of recent faecal pollution. The results of this part of the study are in line with those conducted elsewhere, which showed that MST, in identifying the biological source of pollution, is a robust technology.

These results validated the use of these markers for use in Ireland. The technology was subsequently applied to the Dargle catchment, which is characterised by contrasting land use. The initial use of MST was to analyse water samples of the Dargle and its main tributaries, at the point at which these discharge into the Dargle. Using qualitative MST (end-point PCR) as a tool, the authors identified the main faecal pollution sources in the catchment. These results were in agreement with land use. For example, the upstream reaches of the Dargle, in which there is little or no human activity, only identified ruminant pollution, whereas an urban stream was almost exclusively impacted on by human faecal pollution. The use of MST in its qualitative form (end-point PCR) is therefore a useful method in identifying the main biological sources of pollution in a catchment.

This catchment study was refined by employing a quantitative method (qPCR), which determines the levels of the MST marker in water. The advantage of this approach is that variations in pollution levels can be determined, which are not obvious when using a qualitative method. Using this quantitative approach, it was shown that the amount of human pollution, as measured by the levels of the human marker in the Dargle, differs significantly, identifying the Kilmacanogue and Swan Rivers as the most heavily impacted on by human pollution.

This quantitative method is very useful when employed as a forensic tool, not only in determining the biological origin of faecal contamination, but also its geographical origin. This was demonstrated in two tributaries of the Dargle, the Kilmacanogue and Swan Rivers, which have the highest levels of FIB in the catchment. While qualitative MST only demonstrated that there is human pollution, quantitative MST of water samples taken along the course of these rivers clearly identified the geographical location where the levels of human pollution were increased. This quantitative approach is therefore an extremely useful tool in pinpointing sources of pollution in a catchment, which may be caused by sewer misconnections or faulty septic tanks. In a similar fashion, other MST markers can be used to pinpoint the source of pollution by agricultural activities and by wildlife. Since the authors determined the levels of MST markers in faeces of humans and a range of animals, they were able to estimate the minimal amount of faecal matter that is required to explain the MST levels in a water sample. This was demonstrated in the Swan River, showing that at least 48 kg per day of faecal-derived matter is deposited in the Swan River, most of which occurred in between Hollybrook Park and the Wheatfield Estate.

In summary, this study shows that MST, in both its quantitative and qualitative form, is a useful tool in determining both the biological and geographical origins of faecal contamination. While this study was carried out in a river catchment, the methodology can equally be employed in marine bathing waters and beaches, as well as in ground and well waters. This methodology is, in the authors' view, an essential tool in assisting water quality managers to decide on the best approach to ameliorate poor water quality. However, it does not replace traditional water quality testing using faecal indicator bacteria.

Recommendations

The authors recommend that a two-tiered approach is used in the analysis of faecal contamination of water bodies. Initially, water quality testing using faecal indicator bacteria will reveal if a water body is contaminated, and whether this is of sufficient concern that corrective measures have to be taken. Subsequently, microbial source tracking should be used to identify the biological and geographical origins of faecal pollution, which will assist water quality managers in deciding on the most appropriate corrective measures.

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Abbreviations and Acronyms

16S rRNA	16S ribosomal RNA
AllBac	PCR primer to amplify Bacteriodales 16S rRNA genes
CBR	County Brook
CF128	Ruminant MST Marker
CFU	Colony Forming Unit
CI	Confidence Interval
CowM3	Cow MST Marker
CSO	Outfall of the Enniskerry Wastewater Treatment Plant
DRG	Dargle
EPA	Environmental Protection Agency
FIB	Faecal Indicator Bacteria
gc	Gene copies
GCR	Glencree River
GCU	Glencullen River
GIS	Geographic Information System
HF183	Human MST Marker
Ho597	Horse MST Marker
ICREW	Improvement of Coastal and Recreational Waters, an EU Interreg IIIb funded project focusing on the revised Bathing Water Directive.
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KGH	Killough River
KMG	Kilmacanogue
MPN	Most Probable Number
MST	Microbial Source Tracking
OSI	Ordnance Survey Ireland
Ovmito	Sheep MST Marker
PCR	Polymerase Chain Reaction
PF163	Pig MST Marker
Pig-2-Bac	Pig MST Marker
PMA	Propidium monoazide
Pomito	Pig MST Marker
PS/TS	Positive Sample/Total Sample
qPCR	Quantitative PCR
SWN	Swan River
T_{90}	Time required to reduce the number of bacteria by 90%
UCD	University College Dublin
WWTP	Wastewater Treatment Plant
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

AN GHNÍOMHAIREACHT UM CHAOMHNÚ COMHSHAOIL

Tá an Ghníomhaireacht um Chaomhnú Comhshaoil (GCC) freagrach as an gcomhshaol a chaomhnú agus a fheabhsú mar shócmhainn luachmhar do mhuintir na hÉireann. Táimid tiomanta do dhaoine agus don chomhshaol a chosaint ó éifeachtaí díobhálacha na radaíochta agus an truaillithe.

Is féidir obair na Gníomhaireachta a roinnt ina trí phríomhréimse:

Rialú: *Déanaimid córais éifeachtacha rialaithe agus comhlíonta comhshaoil a chur i bhfeidhm chun torthaí maithe comhshaoil a sholáthar agus chun díriú orthu siúd nach gcloíonn leis na córais sin.*

Eolas: *Soláthraímid sonraí, faisnéis agus measúnú comhshaoil atá ar ardchaighdeán, spriocdhírithe agus tráthúil chun bonn eolais a chur faoin gcinnteoireacht ar gach leibhéal.*

Tacaíocht: *Bímid ag saothrú i gcomhar le grúpaí eile chun tacú le comhshaol atá glan, táirgiúil agus cosanta go maith, agus le hiompar a chuirfidh le comhshaol inbhuanaithe.*

Ár bhFreagrachtaí

Ceadúnú

- Déanaimid na gníomhaíochtaí seo a leanas a rialú ionas nach ndéanann siad dochar do shláinte an phobail ná don chomhshaol:
- saoráidí dramhaíola (m.sh. láithreáin líonta talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh. déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- an diantalmhaíocht (m.sh. muca, éanlaith);
- úsáid shrianta agus scaoileadh rialaithe Orgánach Géinmhodhnaithe (OGM);
- foinsí radaíochta ianúcháin (m.sh. trealamh x-gha agus radaiteiripe, foinsí tionsclaíocha);
- áiseanna móra stórála peitril;
- scardadh dramhuisce;
- gníomhaíochtaí dumpála ar farraige.

Forfheidhmiú Náisiúnta i leith Cúrsaí Comhshaoil

- Clár náisiúnta iniúchtaí agus cigireachtaí a dhéanamh gach bliain ar shaoráidí a bhfuil ceadúnas ón nGníomhaireacht acu.
- Maoirseacht a dhéanamh ar fhreagrachtaí cosanta comhshaoil na n-údarás áitiúil.
- Caighdeán an uisce óil, arna sholáthar ag soláthraithe uisce phoiblí, a mhaoirsiú.
- Obair le húdaráis áitiúla agus le gníomhaireachtaí eile chun dul i ngleic le coireanna comhshaoil trí chomhordú a dhéanamh ar líonra forfheidhmiúcháin náisiúnta, trí dhíriú ar chiontóirí, agus trí mhaoirsiú a dhéanamh ar leasúchán.
- Cur i bhfeidhm rialachán ar nós na Rialachán um Dhramhthrealamh Leictreach agus Leictreonach (DTLL), um Shrian ar Shubstaintí Guaiseacha agus na Rialachán um rialú ar shubstaintí a ídíonn an ciseal ózóin.
- An dlí a chur orthu siúd a bhriseann dlí an chomhshaoil agus a dhéanann dochar don chomhshaol.

Bainistíocht Uisce

- Monatóireacht agus tuairisciú a dhéanamh ar cháilíocht aibhneacha, lochanna, uiscí idirchriosacha agus cósta na hÉireann, agus screamhuiscí; leibhéil uisce agus sruthanna aibhneacha a thomhas.
- Comhordú náisiúnta agus maoirsiú a dhéanamh ar an gCreat-Treoir Uisce.
- Monatóireacht agus tuairisciú a dhéanamh ar Cháilíocht an Uisce Snámha.

Monatóireacht, Anailís agus Tuairisciú ar an gComhshaol

- Monatóireacht a dhéanamh ar cháilíocht an aeir agus Treoir an AE maidir le hAer Glan don Eoraip (CAFÉ) a chur chun feidhme.
- Tuairisciú neamhspleách le cabhrú le cinnteoireacht an rialtais náisiúnta agus na n-údarás áitiúil (m.sh. tuairisciú tréimhsiúil ar staid Chomhshaol na hÉireann agus Tuarascálacha ar Tháscairí).

Rialú Astaíochtaí na nGás Ceaptha Teasa in Éirinn

- Fardail agus réamh-mheastacháin na hÉireann maidir le gáis cheaptha teasa a ullmhú.
- An Treoir maidir le Trádáil Astaíochtaí a chur chun feidhme i gcomhair breis agus 100 de na táirgeoirí dé-ocsaíde carbóin is mó in Éirinn

Taighde agus Forbairt Comhshaoil

- Taighde comhshaoil a chistiú chun brúnna a shainaithint, bonn eolais a chur faoi bheartais, agus réitigh a sholáthar i réimsí na haeráide, an uisce agus na hinbhuanaitheachta.

Measúnacht Straitéiseach Timpeallachta

- Measúnacht a dhéanamh ar thionchar pleananna agus clár beartaithe ar an gcomhshaol in Éirinn (m.sh. mórphleananna forbartha).

Cosaint Raideolaíoch

- Monatóireacht a dhéanamh ar leibhéil radaíochta, measúnacht a dhéanamh ar nochtadh mhuintir na hÉireann don radaíocht ianúcháin.
- Cabhrú le pleananna náisiúnta a fhorbairt le haghaidh éigeandálaí ag eascairt as taismí núicléacha.
- Monatóireacht a dhéanamh ar fhorbairtí thar lear a bhaineann le saoráidí núicléacha agus leis an tsábháilteacht raideolaíochta.
- Sainseirbhísí cosanta ar an radaíocht a sholáthar, nó maoirsiú a dhéanamh ar sholáthar na seirbhísí sin.

Treoir, Faisnéis Inrochtana agus Oideachas

- Comhairle agus treoir a chur ar fáil d'earnáil na tionsclaíochta agus don phobal maidir le hábhair a bhaineann le caomhnú an chomhshaoil agus leis an gcosaint raideolaíoch.
- Faisnéis thráthúil ar an gcomhshaol ar a bhfuil fáil éasca a chur ar fáil chun rannpháirtíocht an phobail a spreagadh sa chinnteoireacht i ndáil leis an gcomhshaol (m.sh. Timpeall an Tí, léarscáileanna radóin).
- Comhairle a chur ar fáil don Rialtas maidir le hábhair a bhaineann leis an tsábháilteacht raideolaíoch agus le cúrsaí práinnfhreagartha.
- Plean Náisiúnta Bainistíochta Dramhaíola Guaisí a fhorbairt chun dramhaíl ghuaiseach a chosc agus a bhainistiú.

Múscailt Feasachta agus Athrú Iompraíochta

- Feasacht chomhshaoil níos fearr a ghiniúint agus dul i bhfeidhm ar athrú iompraíochta dearfach trí thacú le gnóthais, le pobail agus le teaghlaigh a bheith níos éifeachtúla ar acmhainní.
- Tástáil le haghaidh radóin a chur chun cinn i dtithe agus in ionaid oibre, agus gníomhartha leasúcháin a spreagadh nuair is gá.

Bainistíocht agus struchtúr na Gníomhaireachta um Chaomhnú Comhshaoil

Tá an ghníomhaíocht á bainistiú ag Bord lánaimseartha, ar a bhfuil Ard-Stiúrthóir agus cúigear Stiúrthóirí. Déantar an obair ar fud cúig cinn d'Oifigí:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig Forfheidhmithe i leith cúrsaí Comhshaoil
- An Oifig um Measúnú Comhshaoil
- An Oifig um Cosaint Raideolaíoch
- An Oifig Cumarsáide agus Seirbhísí Corparáideacha

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag comhaltaí air agus tagann siad le chéile go rialta le plé a dhéanamh ar ábhair imní agus le comhairle a chur ar an mBord.

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Identifying the Biological and Geographical Origins of Faecal Contamination

Authors: Elisenda Ballesté and Wim G. Meijer

Identifying Pressures

Faecal contamination of water bodies, including rivers, ground and well waters and designated bathing waters poses a risk to human health and may cause significant economic loss. A major obstacle to implementing corrective measures is a lack of validated techniques to identify the biological and geographical origins of faecal contamination.

Informing Policy

The EU Water Framework Directive requires that when environmental objectives are unlikely to be achieved, Member States have to investigate the causes of possible failure. The Bathing Water Directive calls for the identification and assessment of causes of pollution that might affect bathing waters and impair bather's health. This study provides a methodology to address these aspects of the Water Framework and Bathing Water Directives.

Developing Solutions

This study validates a number of Microbial Source Tracking markers for use in Ireland and demonstrates their usefulness in identifying the biological and geographical origins of faecal contamination. The information provided by application of these tools will assist Irish water quality managers to identify appropriate measures to reduce faecal contamination of water bodies.

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